**REFERENCE ONLY**

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# Analysis of CD45 variants effecting alternative splicing

**Sally Anne Boxall**

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ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

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# Abstract

The CD45 (leukocyte common) antigen is a haemopoietic cell specific tyrosine phosphatase essential for antigen receptor signalling in lymphocytes. Multiple isoforms of CD45 are expressed in a cell type and activation-specific manner, but the exact function of the different isoforms remains obscure. In humans, naive T cells express high molecular weight isoforms (containing CD45RA), but following activation switch to expression of low molecular weight (CD45R0 and CD45RB) isoforms. Human CD45 variant alleles which alter CD45 isoform expression have been identified and associated with infectious and autoimmune diseases.

Two contrasting allelic variants have been analysed. The exon 4 77G allele is present at a low frequency in Caucasoids and prevents splicing from high to low molecular weight isoforms. An increased frequency of this allele is found in multiple sclerosis, HIV and Hepatitis C infected individuals. The exon 6 138G allele is found at a high frequency in Far Eastern populations and promotes splicing towards low molecular weight isoforms. This allele is protective in Graves' disease and Hepatitis B infection. Both alleles are associated with altered phenotype and *in vitro* functional response of T cells. Similarly CD45 transgenic mice exhibit altered T cell phenotype and function. These data demonstrate that subtle changes in isoform expression lead to an alteration in cell phenotype and that both combinations of isoforms and the total level of expression are important for CD45 function.

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## Common Abbreviations

| | |
|---|---|
| APC | Antigen presenting cell(s) |
| APC | Allophycocyanin |
| ASF | Alternative splicing factor |
| BBP | Branch point binding protein |
| BCR | B cell receptor |
| bp | Base pairs |
| BSA | Bovine serum albumin |
| cDNA | Complementary deoxyribonucleic acid |
| cpm | Count per minute |
| CTL | Cytotoxic T lymphocyte |
| Da | Dalton |
| DAG | Diacyl glycerol |
| dATP | Deoxyadenosine triphosphate |
| DC | Dendritic cell |
| DEPC | Diethyl pyrocarbonate |
| DMEM | Dulbecco's modified Eagle's medium |
| DMSO | Dimethylsulphoxide |
| DNase | Deoxyribonuclease |
| DNA | Deoxyribonucleic acid |
| dNTP | Deoxynucleotide triphosphate |
| EBV | Epstein Barr virus |
| EDTA | Diaminoethanetetra acetic acid |
| ERE | Exonic recognition element |
| ESE | Exonic splicing enhancer |
| ESS | Exonic splice silencer |
| FACS | Fluorescence activated cell sorter |
| FCS | Foetal calf serum |
| FITC | Fluorescein isothiocyanate |
| g | gram or gravitational force |

| | |
|---|---|
| HBV | Hepatitis B virus |
| HCV | Hepatitis C virus |
| HIV | Human immunodeficiency virus |
| HLA | Human leukocyte antigens |
| HS | Human AB Serum |
| ICAM | Intracellular adhesion molecule |
| IFN | Interferon |
| Ig | Immunoglobulin |
| IL | Interleukin |
| $IP_3$ | Inositol-1,4,5,triphosphate |
| ITAM | Immunoglobulin receptor family tyrosine-based activation motif |
| kb | Kilobase |
| kDa | Kilodalton |
| LAT | Linker of activation in T cells |
| LCA | Leukocyte common antige (CD45) |
| LFA | Lymphocyte functional antigen |
| mM | millimolar |
| M | molar |
| mA | milliamp |
| mAb | monoclonal antibody |
| mg | milligram |
| µg | microgram |
| MHC | Major histocompatibility complex |
| ml | millilitre |
| µl | microlitre |
| mRNA | messenger ribonucleic acid |
| ng | nanogram |
| NK cell | Natural killer cell |
| PBMC | Peripheral blood mononuclear cells |
| PBS | Phosphate buffered saline |
| PCR | Polymerase chain reaction |
| PE | Phycoerythrin |
| PERCP | Peridinin chlorophyll protein |

| | |
|---|---|
| PHA | phytohaemagglutinin |
| $PIP_2$ | Phosphoinositol diphosphate |
| PKC | Protein kinase C |
| PLC | Phospholiapse C |
| PPD | Purified protein derivative (of *M. tuberculosis*) |
| PRR | Pattern recognition receptor |
| PTK | Phosphotyrosine kinase |
| PTP | Phosphotyrosine phosphatase |
| RNA | Ribonucleic acid |
| RNase | Ribonuclease |
| RPE-CY5 | R-phycoerythrin cyanine 5 |
| rpm | revolutions per minute |
| RRM | RNA recognition motif |
| RT | Reverse transcriptase |
| SCID | Severe combined immunodeficiency |
| SH2/3 | Src-homologous domsin 2/3 |
| snRNP | Small nuclear ribo-nucleo-particle |
| SR-domain | Serine-arginine rich domain |
| SRrps | SR-related protein |
| SV40 | Simian virus type 40 |
| TAE | Tris acetate EDTA |
| Taq | Thermus aquaticus |
| TBE | Tris borate EDTA |
| Tc | T cytotoxic |
| TCR | T cell receptor |
| Tg | Transgenic |
| Th1 | T helper type 1 cell |
| Th2 | T helper type 2 cell |
| TNF | Tumour necrosis factor |
| TT | Tetanus toxoid |
| v | volt |
| ZAP | ζ-chain-associated protein |

# Outline of Thesis

The leukocyte common antigen, CD45 is a haemopoietic cell specific protein tyrosine phosphatase, comprising up to 10% of the cell surface. CD45-deficient humans and mice are severely immunodeficient and it has been shown to be essential for efficient antigen receptor signalling in lymphocytes. Multiple isoforms of CD45 are expressed in a cell type and activation-specific manner. In humans, activation of naïve T cells switches expression from high to low molecular weight isoforms. However, despite being tightly regulated the exact function of the different CD45 isoforms remains obscure.

The aim of this thesis is to investigate the function of the different CD45 isoforms, specifically focusing on how the expression of alternatively spliced isoforms affects the function of lymphocytes. We will utilise the existence of human CD45 variant alleles to determine the effects of altered isoform expression on T cell phenotype and function and investigate the mechanisms responsible for altered immune function.

This thesis is divided into eight chapters which are outlined as follows. Chapter one contains four sections, the first section being a brief overview of the human immune system with particular reference to the phenotype and function of naïve and memory T cells. Section 1.2 provides an overview of the CD45 molecule, and our current understanding of its functional mechanisms. Section 1.3 focuses specifically on human CD45 polymorphisms and their disease associations, whilst section 1.4 outlines the detailed objectives of this study. The methods employed throughout this thesis are described in Chapter 2.

In Chapter 3, CD45 isoform expression on leukocytes and T cell phenotype is characterised in CD45 variant individuals. Functional responses, both proliferative responses and cytokine production of T cells from CD45 variant individuals to various stimuli are described in Chapter 4. Minigenes are used in Chapter 5 to analyse the molecular mechanisms responsible for altered isoform expression in 138G variant individuals. Chapter 6 briefly describes the CD45 isoform expression in HIV seropositive individuals with novel CD45 polymorphisms. A transgenic mouse model

has been created which approximates the abnormal CD45 splicing observed in humans with the C77G polymorphism. The T cell phenotype and function of these mice is described in Chapter 7. Finally the results of the whole investigation are brought together and discussed in Chapter 8 along with suggestions of areas for further study.

# CHAPTER 1

## Introduction

### 1.1.0 The immune system

Our environment contains a large variety of infectious microbes including bacteria, viruses, fungi, protozoa and multi-cellular parasites. These can all cause infection, which if left uncontrolled may eventually kill the host. The body has therefore had to develop a defence system which is not only capable of fighting a variety of pathological invaders, but can distinguish these pathogens (referred to as non-self) from its own cells (self).

Micro-organisms exist in many different forms, requiring a wide variety of immune responses to deal with the various infections. As an exterior defence, skin epithelial cells provide a strong physical and chemical barrier to most organisms. However, many invaders gain access across the epethelia of the gastrointestinal or urogenital tracts, can infect the nasopharynx and lungs, or can even directly enter the blood. The site of infection and type of pathogen, whether it is intra- or extra-cellular, will largely determine which immune response will be effective.

### 1.1.1 The innate immune response

The first part of any immune response is the recognition of the pathogen. This is followed by a suitable reaction to eliminate it. Specificity and mechanism of response can be used to distinguish two different but interrelated types of immunity, that of the innate and adaptive immune systems. The innate system provides the first line of defence, a rapid but non-specific response to pathogens. These responses may be humoral, including the complement system, or cellular, such as NK cells and phagocytes (e.g. granulocytes, macrophages and dendritic cells).

The innate system is able to recognise the pathogen as 'foreign' or 'non-self'. Cells of the innate system operate through germ-line encoded receptors called pattern recognition receptors (PRRs). These receptors recognise pathogen associated molecular patterns (PAMPS), conserved molecular structures shared by a variety of pathogens, which may include polysaccharides, proteins, lipids and nucleic acid motifs, such as bacterial CpG islands (Medzhitov and Janeway, 2000). A variety of innate receptors have been identified including the mannose receptor which recognizes structures on the surface of bacteria, yeasts and parasites, triggering phagocytosis and opsonisation and initiating inflammatory responses (Martinez-Pomares and Gordon, 1999b; Gordon, 2002). Myeloid cells, including macrophages and dendritic cells, also express scavenger receptors, which recognise molecules such as lipoteichoic acid and LPS and facilitate the uptake and clearance of apoptotic cells.

Many cell types use a variety of Toll like receptors (TLRs) to recognise different features of bacterial and viral products. TLRs induce a range of defence mechanisms dependant upon the stimulus and receptor usage. There is a general induction of iNOS (inducible Nitric Oxide Synthase) and corresponding induction of Nitric Oxide (NO) which has antimicrobial properties. TLR signalling also induces the production of cytokines and co-stimulatory molecules including TNFα (Tumour necrosis factor α), which can activate macrophages, causing them to release defensins and facilitating the adaptive immune response.

The innate response is amplified through the release of various soluble factors, such as defensins, cytokines, chemokines and complement. The complement system is a group of about 20 serum proteins whose overall function is the control of inflammation. Activation of the complement cascade can result in; direct killing of micro-organisms, opsonization of target cells, activation of phagocytes and augmentation of antibody responses (Carroll, 1998).

NK cells, which can comprise up to 15% of peripheral blood lymphocytes, are an important link between the innate and adaptive immune responses. They have a role in controlling viral infections and tumours, by detecting cells expressing low or no MHC class 1, an evasion tactic employed by many viruses (Cerwenka and Lanier, 2001).

Once activated they have direct cytolytic activity (using the same mechanisms as CD8+ T cells) and release cytokines which selectively induce Th1 T cell responses.

### 1.1.2 The adaptive immune response

The innate immune response provides a rapid, but non-specific response to pathogens. This is important, but limited, as it is not capable of recognising specific pathogen peptides and cannot generate immunological memory. However, activation of an innate response primes the adaptive immune response, which is capable of generating specific responses and can confer lifelong protection against previously encountered antigens.

Tissue resident and immature DCs are recruited to the site of an infection, where they take up and process antigen. They then mature and migrate to the lymphoid tissues, where they interact with both T cells and B cells. Some native antigen may also migrate through the lymphatic system to the lymph nodes, where it can be taken up by follicular DCs and presented to B cells. B cells recognise antigen via the B cell antigen receptor, the specificity of which is determined by recombination of germ line genes of the heavy and light chains during development. Some B cells will internalise the antigen and act as antigen presenting cells (APCs) for T cells. After activation and clonal expansion some B cells will differentiate into plasma cells and produce antibodies.

The specificity of the adaptive immune response is due to positive and negative selection events during T cell development, coupled with receptor rearrangement. This results in a variety of T cell clones, each characterised by a specific receptor. Activation of T cells requires two signals: One signal is provided by the antigen-MHC complex on the APCs, with the second signal provided by co-stimulatory molecules. Expansion of antigen specific T cell clones occurs after activation. Some of the cells from each clone will become effector cells, before dying by apoptosis, whilst others will become the memory cells which are responsible for secondary responses.

### 1.1.3 Cells of the immune system

Leukocytes, the cellular components of the immune system, are derived from pluripotent haemopoietic stem cells in the bone marrow. These stem cells give rise to two lineages of cells in the blood via myeloid and lymphoid progenitors (Figure 1.1). Myeloid progenitors give rise to polymorphonuclear granulocytes (including basophils, neutrophils and eosinophils), erythroid progenitors and monocytes. Found in both blood and tissues, granulocytes are important in the phagocytosis of pathogens. Monocytes are found in the blood, but differentiate into macrophages in the tissues. These cells are important as both phagocytic cells and antigen presenting cells, displaying antigens to the adaptive immune response. Derived from both myeloid and lymphoid progenitors, dendritic cells are often referred to as professional antigen presenting cells. They are highly efficient in the uptake of antigen and presentation of it to cells of the adaptive immune system. Although also derived from the same haemopoietic stem cells, Erythrocyes (red blood cells) and platelets are not directly involved in the immune response, but platelets do have an important role in blood clotting and inflammation.

Lymphoid progenitors give rise to lymphocytes; T cells, B cells and NK cells. Unlike other lymphocytes, NK cells do not have antigen specific receptors. Lymphocytes fall into two major subgroups, T cells (thymus derived cells) and B cells.

B cells, named for their development in the Bursa of Fabricius in birds, mature in the foetal liver or bone marrow of humans. B cells migrate between the blood and lymphoid tissues and are responsible for mediating humoral responses. Activation of B cells through the B cell receptor (BCR) results in the generation of plasma cells, which produce antibodies (immunoglobulin molecules) that may inactivate bacteria by neutralisation, opsonisation or activating compliment. B cell receptors recognise native antigen, which can be protein or specific peptide, but may also be carbohydrates, lipids or synthetic molecules. T cell receptors are only capable of recognising specific antigenic peptides. Both B and T cells can form memory cells, which respond more efficiently on re-exposure to the same antigen.

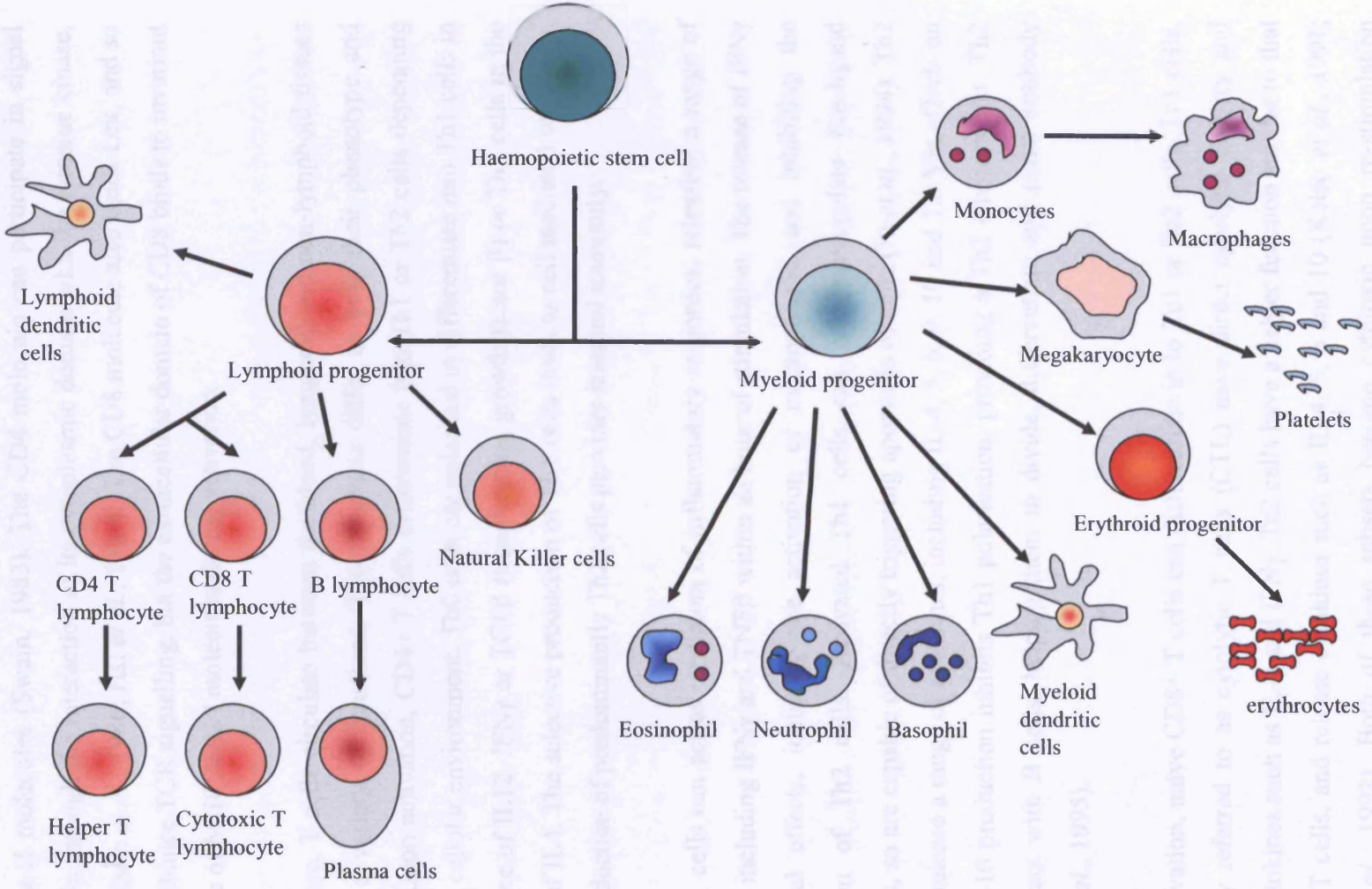



**Figure 1.1 Schematic representation of the differentiation of cells of the immune system.**

T cells originate in the bone marrow, before migrating to the thymus where they develop and mature and differentiate into CD4+ and CD8+ T cells. During antigen recognition and activation, the CD4 extracellular domain binds the invariant part of the MHC class II molecules (Swain, 1983). The CD4 molecule can participate in signal transduction through the interaction of its cytoplasmic domain with the tyrosine kinase, Lck (Samelson *et al.*, 1986; Hsi *et al.*, 1989). The CD8 molecule also binds Lck, and so can also enhance TCR signalling, but the extracellular domain of CD8 binds to invariant parts of the of MHC class I molecules during activation.

Once mature, T cells circulate between the blood, lymphoid and non-lymphoid tissues and have a variety of functions. T cell subsets differ in both their phenotype and function. Upon activation, CD4+ T cells differentiate into Th1 or Th2 cells depending upon their cellular environment. Th0 cells are polarised to differentiate into Th1 cells in the presence of IL12, IFNγ or TGFβ (transforming growth factor β) or Th2 cells in the presence of IL4. The selective production of Th1 cells leads to cell mediated immunity, whilst production of predominantly Th2 cells provides humoral immunity.

Some Th1 cells can act as mediators of inflammatory responses, releasing a range of cytokines, including IFNγ and TNFβ within an hour of stimulation. The release of IFNγ has several effects, including the activation of macrophages, and inhibiting the polarisation of Th2 cells. Activated Th1 cells can also up-regulate Fas-ligand expression, so are capable of directly triggering apoptosis of cells (Ju *et al.*, 1994). Th2 cells also release a range of cytokines, including IL-4, 5, 6, 9, 10 and 13. Via effects on APCs, IL-10 production inhibits Th1 polarisation, providing a Th2 environment. Th2 cells interact with B cells, helping them to divide, differentiate and make antibody (Mond *et al.*, 1995).

Upon activation, naïve CD8+ T cells can differentiate in to Tc1 or Tc2 cells. Tc1 cells, commonly referred to as cytolytic T cells (CTL) have direct cytolytic activity and release cytokines such as IL-2 and IFNγ. Tc2 cells have a helper function similar to that of CD4+ T cells, and release cytokines such as IL-4, 5, 6 and 10 (Kelso *et al.*, 1991; Seder *et al.*, 1992). Both of these subsets become cytotoxic upon re-stimulation (Cerwenka *et al.*, 1998). CTL are important mediators of cell cytotoxicity, killing cells

infected with viruses or intracellular pathogens and tumour cells. These cells can directly kill the infected cells through a number of mechanisms, including the release of lytic granules into infected cells (such as perforin or granzyme) and the induction of Fas mediated apoptosis.

### 1.1.4 The T cell receptor

The structure of the TCR is highly conserved. It is a heterodimer of two transmembrane glycoproteins, either the α,β or γ,δ chains (Figure 1.2). In humans, around 95% of peripheral T cells express the αβ TCR, whilst the remainder (1-10%) express the γδ TCR. These cells differ in the way that they recognise antigen and are thought to have a role in the recognition of non-classical MHC molecules and heat shock proteins (Janeway *et al.*, 1988; O'Brien *et al.*, 1989; Moss *et al.*, 1992).

The transmembrane glycoproteins of the TCR have an amino-terminal extracellular domain, containing variable and constant regions. They also have a short linker joining to the membrane spanning domain and a short cytoplasmic tail. The functional TCR complex which is expressed on the surface of cells is an association between these variant TCRαβ chains and the invariant TCRζ chain and CD3 (γ,δ and ε) chains. The TCR chains recognise the antigen, whilst the TCRζ and CD3 subunits transduce the signal via immuno-receptor tyrosine-based motifs (ITAMS) in their cytoplasmic domains (Weiss and Littman, 1994).

The TCR chains only recognise specific peptides bound to self-MHC molecules. Each TCR chain comprises variable (V), diversity (D), joining (J) and constant (C) regions. Each of these regions is encoded by many genes, which can be rearranged during T cell development. This, along with the addition and removal of nucleotides during recombination, gives rise to a vast number of different αβTCR combinations, generating the great diversity of TCR specificity.

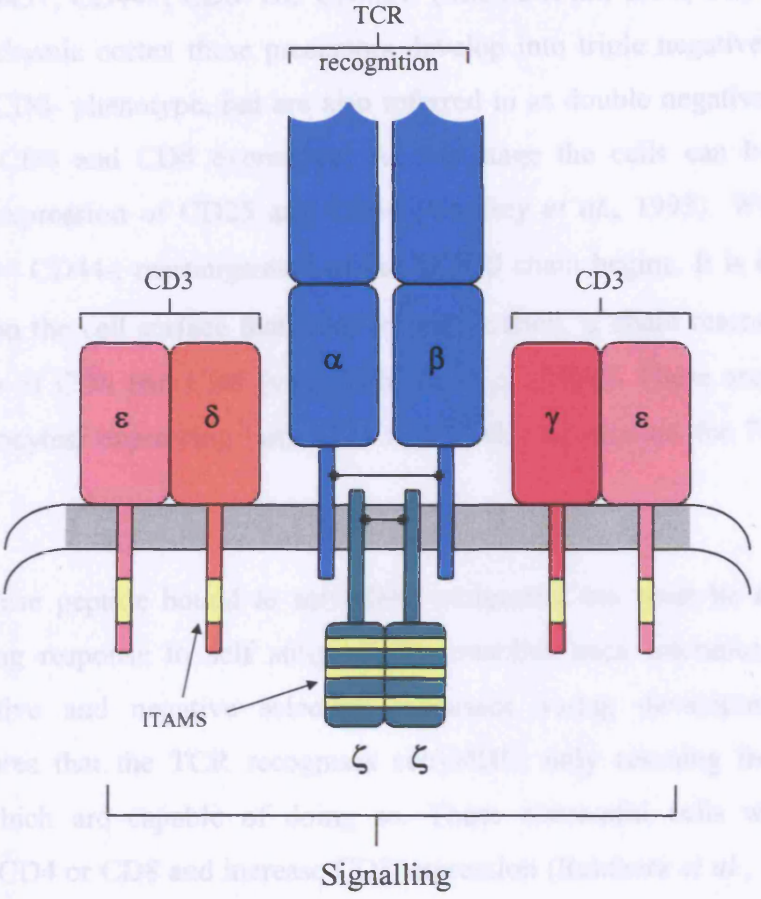



**Figure 1.2 Schematic representation of the T cell receptor complex.** The T-cell receptor complex is made up of antigen-recognition proteins and invariant signalling proteins. The TCR α:β heterodimer recognises and binds peptide bound to MHC, but cannot signal to the cell directly. Signalling is carried out through the four CD3 signalling chains and the associated ζ chains. Each CD3 chain has one ITAM (represented by the yellow segment), whilst each ζ chain contains 3 ITAMs.

### 1.1.5 T cell development

T cell progenitors migrate from the bone marrow to the thymus, where they mature, undergoing TCR rearrangement and selection events (Figure 1.3). These precursor cells are HSA+, CD43+, CD44+, CD8- and CD4low (Michie *et al.*, 1998; Schwarzler *et al.*, 2001). In the thymic cortex these precursors develop into triple negative cells with a CD3-, CD4-, CD8- phenotype, but are also referred to as double negative cells due to their lack of CD4 and CD8 expression. At this stage the cells can be subdivided according to expression of CD25 and CD44 (Godfrey *et al.*, 1993). When the cells become CD25+ CD44-, rearrangement of the TCR β chain begins. It is expression of the pre TCR on the cell surface that leads to proliferation, α chain rearrangement and the expression of CD4 and CD8 (von Boehmer *et al.*, 1988). These are now double positive thymocytes, expressing both CD4 and CD8, and account for 70-80% of all thymocytes.

T cells recognise peptide bound to self-MHC molecules, but must be able to do so without making response to self antigens. To establish such tolerance, thymocytes undergo positive and negative selection processes during development. Positive selection ensures that the TCR recognises self-MHC, only rescuing from apoptosis those cells which are capable of doing so. These successful cells will then lose expression of CD4 or CD8 and increase CD3 expression (Reinherz *et al.*, 1980; Roehm *et al.*, 1984; Lopez-Botet and Moretta, 1985; Saito and Watanabe, 1998). Some of these single positive cells may still bind self-peptides. Therefore the thymocytes are negatively selected, via Fas induced apopotosis (Goldrath and Bevan, 1999). Around 95% of the double positive thymocytes die during development. The remaining 5% of cells are MHC restricted, self-tolerant, single positive cells, whose TCR has the appropriate affinity and avidity for peptide-MHC complexes. These cells can expand intrathymically to produce a repertoire of cells that will efficiently target foreign antigens without damaging the host. The T cells then leave the thymus, circulating between the blood and lymph tissues, until they encounter antigen in the lymph nodes.

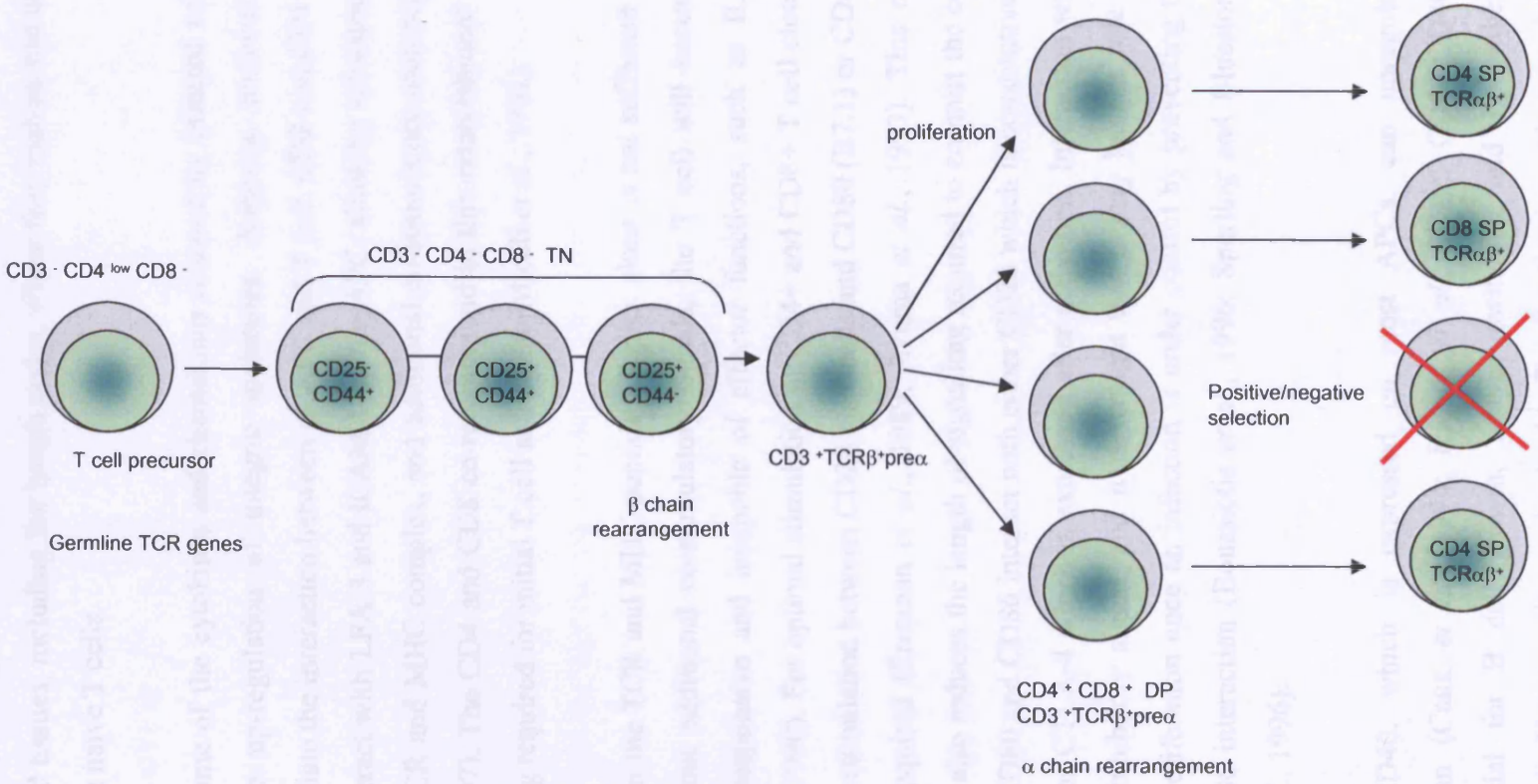



**Figure 1.3 Schematic representation of the T cell development in the thymus.** T cells progress through the triple negative stage, with varying expression of CD25 and CD44. As the cells become CD25+ CD44-, TCR rearrangement begins, and is completed at the double positive stage, where the thymocytes express both CD4 and CD8. Cells which survive the negative and positive selection processes, become CD4+ or CD8+ T cells. Cells which are not successfully selected die by apoptosis.

### 1.1.6 Activation of T cells.

After taking up antigen, changes in chemokine receptor expression cause DCs to migrate to the lymphoid tissues, including the lymph nodes, where they mature and can interact with circulating naïve T cells.

During an infection, some of the cytokines and chemokines released by infected cells and APCs trigger the up-regulation of integrin molecules. Adhesion molecules, including integrins, initiate the interaction between an APC and T cell. CD2 and CD11a (LFA-1) on T cells interact with LFA-3 and ICAM-1 on the APC, allowing subsequent contact between the TCR and MHC complex, and additional co-stimulatory molecules (Shaw and Dustin, 1997). The CD4 and CD8 co-receptors aid in this establishment of contact, as well as being required for initial T cell activation (Miceli *et al.*, 1991).

The interaction between the TCR and MHC-peptide complex alone is not sufficient to activate T cells. Without additional co-stimulatory signals, the T cell will become anergic, becoming unresponsive and incapable of effector functions, such as IL-2 production (Schwartz, 1990). For optimal stimulation of CD4+ and CD8+ T cell clones and isolated T cells, co-stimulation between CD28 on T cells and CD80 (B7.1) or CD86 (B7.2) on APCs is required (Freeman *et al.*, 1989; Azuma *et al.*, 1993). This co-stimulatory interaction also reduces the length of signalling required to commit the cell to proliferation. Both CD80 and CD86 interact with either CD28 which is constitutively expressed on T cells, or CTLA-4 which is expressed after activation. Interaction with CTLA-4 provides an inhibitory signal, and it is thought that it may have a role in switching off T cell proliferation once an infection is under control by preventing the positive CD28-CD80/86 interaction (Boussiotis *et al.*, 1996; Sperling and Bluestone, 1996; Waterhouse *et al.*, 1996).

Signalling through CD40, which is expressed on most APCs, can upregulate CD80/CD86 expression (Caux *et al.*, 1994; Kiener *et al.*, 1995). CD40-CD40L engagement is essential for B cell growth, isotype switching and Ig-synthesis (Banchereau *et al.*, 1994). However, it can also lead to T cell activation, enhancing both Th1 and Th2 type responses (van Essen *et al.*, 1995; Peng *et al.*, 1996). The interaction between CD40L on T cells and CD40 on DCs can result in an up-regulation of MHC

and costimulatory molecule expression and cytokine production by the DCs. This can enable the DC to directly activate naïve CD8+ T cells, in some circumstances overcoming the requirement for CD4+ T cell help (Lanzavecchia, 1998).

### 1.1.7 TCR signalling pathways

Binding of the TCR to the MHC-peptide complex mediates signalling (Figure 1.4) and can result in activation, anergy or apoptosis, depending on the state of T cell differentiation and the influence of co-receptors (Weiss, 1991; Miller and Morahan, 1992; Cohen *et al.*, 2002). The TCR/CD3 complex does not have intrinsic kinase activity, but when bound to antigen it interacts with various membrane-bound proteins including CD4, CD8 and the protein tyrosine phosphatase, CD45. Aggregation of the T cell receptor complex with the appropriate co-receptors aids activation, bringing Lck tyrosine kinases associated with the cytoplasmic domains of the co-receptors together with the ITAMs and other targets associated with the cytoplasmic domains of the TCR complex.

In naive T cells the CD3ε chain is associated with p59fyn (a Src family kinase) whilst the constitutively phosphorylated ζ chain is associated with ZAP-70 via its ITAMS (Wange *et al.*, 1993; Weiss and Littman, 1994). Upon TCR stimulation ζ chain ITAMS are phosphorylated by Lck and fyn, which are activated by CD45. The phosphorylated ζ chain activates ZAP70, which in turn activates LAT (linker of activation in T cells) and SLP-76 (Wardenburg *et al.*, 1996; Zhang *et al.*, 1998). The phosphorylation of LAT leads to an association with several SH2 domain-containing proteins, including growth factor receptor-bound protein 2 (GRB2), phospholipase Cγ1 (PLCγ1), IL2-inducible T-cell kinase (ITK) and the p85 subunit of the lipid kinase phosphatidylinositol 3 kinase (PI3K). GRB2 can then associate with RAS, triggering signalling though the MAP (Mitogen activated protein) kinase cascade. MAPs are serine or threonine kinases, which become sequentially activated by phosphorylation. After activation ERK translocates into the nucleus, where it activates the transcription of the immediate early genes involved in cell division and the IL-2 gene (Cantrell, 1996). LAT also

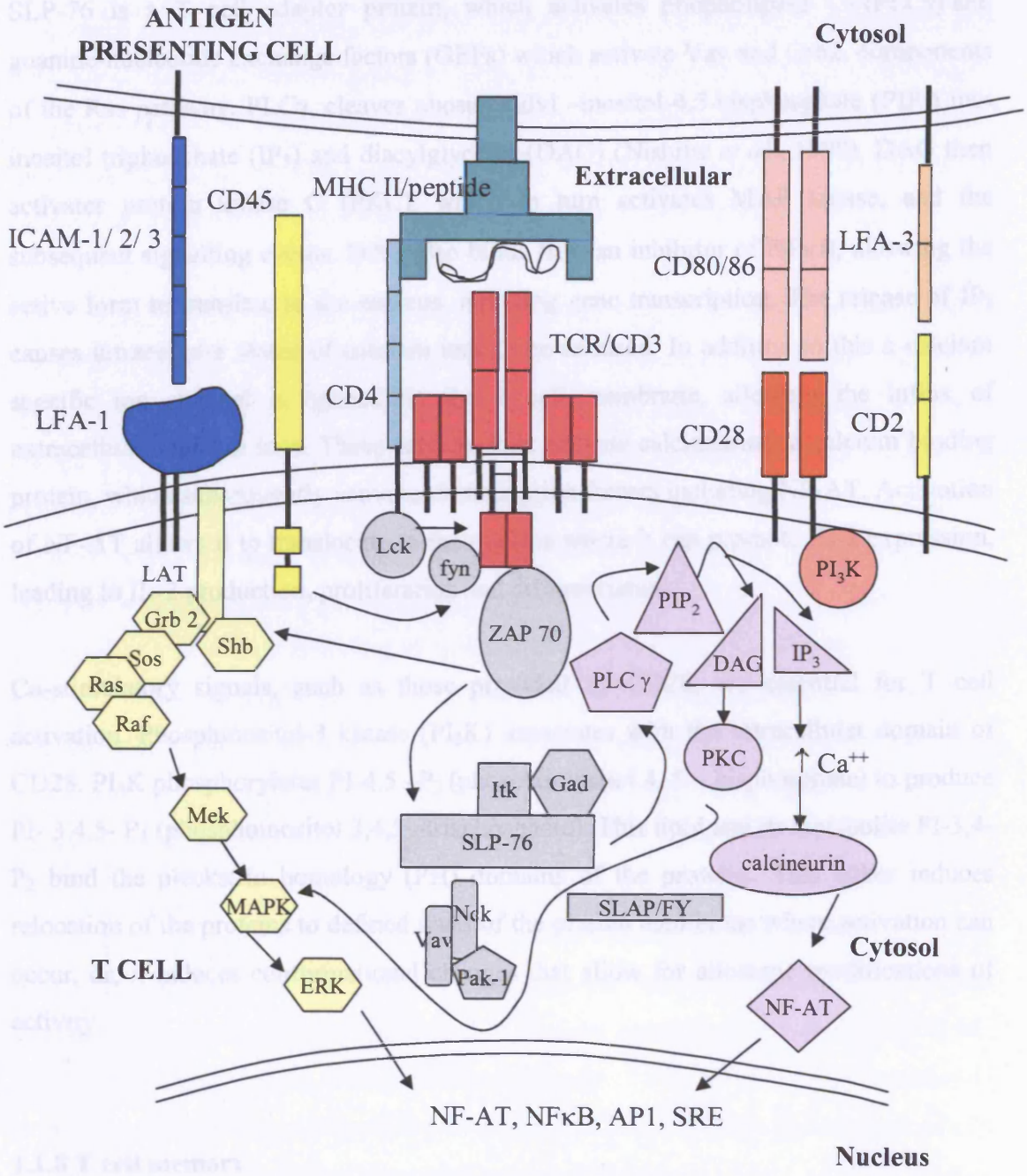



**Figure 1.4 Schematic diagram of CD4+ T cell activation** (not drawn to scale). The interactions between the antigen presenting cell and T cell are shown with the associated signalling events which lead to the activation of the T cell.

constitutively associates with the adaptor GADS, through which it indirectly binds SLP-76.

SLP-76 is a T cell adaptor protein, which activates phopholipase Cγ (PLCγ) and guanine-nucleotide exchange factors (GEFs) which activate Vav and Grb2, components of the Ras pathway. PLCγ, cleaves phosphatidyl –inositol-4,5-bisphosphate ($PIP_2$) into inositol triphosphate ($IP_3$) and diacylglycerol (DAG) (Nishibe *et al.*, 1990). DAG then activates protein kinase C (PKC), which in turn activates MAP kinase, and the subsequent signalling events. DAG also binds IκB, an inhibitor of NFκB, allowing the active form to translate to the nucleus, initiating gene transcription. The release of $IP_3$ causes intracellular stores of calcium ions to be released. In addition to this a calcium specific ion channel is opened on the T cell membrane, allowing the influx of extracellular calcium ions. These calcium ions activate calcineurine, a calcium binding protein, which subsequently activates transcription factors including NF-AT. Activation of NF-AT allows it to translocate to the nucleus where it can promote IL-2 expression, leading to IL-2 production, proliferation and differentiation.

Co-stimulatory signals, such as those provided by CD28, are essential for T cell activation. Phosphinositol-3 kinase ($PI_3K$) associates with the intracellular domain of CD28. $PI_3K$ phosphorylates PI-4,5 $-P_2$ (phosphoinositol 4, 5 – bisphosphate) to produce PI- 3,4,5- $P_3$ (phosphoinositol 3,4,5 –trisphosphate). This lipid and its metabolite PI-3,4-$P_2$ bind the pleckstrin homology (PH) domains of the proteins. This either induces relocation of the proteins to defined areas of the plasma membrane where activation can occur, or, it induces conformational changes that allow for allosteric modifications of activity.

### 1.1.8 T cell memory

In the lymph node, naïve T cells which encounter antigen and the appropriate co-stimulatory molecules undergo clonal expansion, before leaving as effector cells. The majority of cells in each clone (95%) will be short lived, migrating to the site of infection and dying by apoptosis after fulfilling their function. The remaining 5% of the cells will contribute to the maintenance of the memory population that circulate between

the blood and lymph nodes in a relatively resting state until they re-encounter the same antigen. Alterations in these three phases, expansion, contraction (death by apoptosis) and maintenance of memory cells, will determine whether T cell immunity is long or short lived (Ahmed and Gray, 1996). On re-exposure to antigen, cells of the memory population respond more efficiently, with a faster, stronger and more prolonged response (Veiga-Fernandes *et al.*, 2000). This enhanced response is due to a reduced detection threshold and decreased dependency on CD28 mediated co-stimulation in memory cells compared to naïve cells (Fujii *et al.*, 1992; London *et al.*, 2000).

For B cells, memory and effector cells are generated using different pathways, with different signals driving memory or plasma cell formation (Klinman, 1998). However, for T cells the differentiation of memory cells is less clearly understood. It is also complicated by the different methods of phenotypically defining different populations of memory cells. The conventional model of memory T cell generation is through linear differentiation from naïve to effector to memory cells (Farber, 1998). Complementary to this idea is the decreasing potential hypothesis, where after several rounds of division, activated cells lose their potential to become memory cells and are driven to either the effector state or apoptosis. An alternative model to this is that the effector and memory T cells differentiate through different pathways, as observed with B cells and some studies have suggested that memory cells may be directly derived from a subset of naive cells (Liu *et al.*, 2001b).

The maintenance of immunological memory is also a matter for debate. The contentious issue is whether persistent antigen is necessary for the maintenance of memory. Whilst there is some data supporting the idea that it is not necessary (Murali-Krishna *et al.*, 1999; Swain *et al.*, 1999) most experimental models cannot rule out the presence of some persistent antigen. Antigen may be retained on follicular DCs as antigen-antibody complexes for long periods of time, and persistent antigen has been observed in some viral infections, such as Hepatitis B and LCMV (Rehermann *et al.*, 1996; Klenerman *et al.*, 1997). Regardless of the requirement for persistant antigen, homeostasis within the memory population is maintained by a variety of factors, including the presence of certain cytokines, ensuring that the numbers and proportions of the different T cell populations remain constant and the immune system can function normally.

### 1.1.9 Markers for T cell memory

Upon activation T cells change the expression of some cell surface markers, including molecules involved in adhesion, co-stimulation and migration, as well as cytokine expression patterns. These changes in expression are related to the properties of the naïve, effector and memory cell populations, and are often used to define them. Monoclonal antibodies to the leukocyte common antigen, CD45 have been used to define naïve and memory cells. CD45 is a tyrosine phosphatase that is essential for T cell receptor signalling. Alternative splicing of exons 4, 5 and 6 (also known as A, B and C) in the extracellular region of the CD45 molecule leads to the expression of different isoforms.

After mitogenic activation *in vitro*, human T cells were found to lose expression of the high molecular weight isoforms, containing exon A, so termed CD45RA, and gain the expression of the lower molecular weight isoforms, including the 180 kDa null isoform, CD45R0 (Akbar *et al.*, 1988). In an oversimplified manner these subsets have been used to define naïve and memory cells, which do posses very different properties. As both naïve and memory T cells express multiple CD45 isoforms, the terms CD45RA and CD45R0 will be used from now on to describe cells which predominantly express high molecular weight (CD45RA containing) isoforms, and those expressing predominantly low molecular weight isoforms (CD45R0 and CD45RB low).

In the CD4+ cells, the CD45RA+ T cells are small resting cells, which have long telomeres and rarely divide. These CD45RA cells have a more diverse T cell receptor repertoire, and respond to neo-antigens (Young *et al.*, 1997). The CD45R0 cells show evidence of activation, have shorter telomeres and divide quite frequently (Michie *et al.*, 1992; Maini, 1999). Functional studies have also shown that both CD4+ and CD8+ CD45R0+ populations respond to recall antigens (Merkenschlager *et al.*, 1988; Merkenschlager and Beverley, 1989).

However, whilst naïve CD8+ cells express the high molecular weight CD45RA isoforms, the phenotype of memory or activated cells can be variable (Hamann *et al.*, 1997). It has been shown that following prolonged *in vitro* stimulation CD8+ cells can

re-express CD45RA (Warren and Skipsey, 1991), and antigen specific CD8+ CD45RA+ memory cells have been identified in viral infections (Wills *et al.*, 1999; Faint *et al.*, 2001; Dunne *et al.*, 2002). These antigen-experienced cells can still be distinguished from naive cells, by other phenotypic markers, such as CD11a, CD27 and CCR7. Naive CD8+ CD45RA+ cells are predominantly CD27+ and CD28+ and loss of these molecules correlates with repeated stimulation, as is often seen during viral infections. However, there are also some antigen-experienced CD45RA+ cells which express CD27 and not CD28 (Appay *et al.*, 2002; Wills *et al.*, 2002). Whilst CD11a expression is low on naive cells, a population of CD8+ C45RA+ CD11a high cells has been observed which has properties of memory cells and increases with both age and systemic viral infections (Hoflich *et al.*, 1998).

T cells with different effector functions can be defined by alterations in phenotype, characterised by expression patterns of a variety of activation markers and cytokines. Whilst CD45 expression is an extremely useful marker of activation status, to better define naïve and memory populations, other markers also need to be used. The expression levels of adhesion molecules such as CD11a (LFA-1) and isoforms of CD44 are increased on activated T cells. Expression of co-stimulatory molecules, CD27 and CD28, also vary, with CD28 increasing on activation, whilst CD27 is down regulated on chronic activation, and is known to correlate with effector function (De Jong *et al.*, 1992; Hintzen *et al.*, 1993; Hendriks *et al.*, 2000).

Two different populations of memory T cells, central and effector memory, have been further defined by expression of CCR7, a chemokine receptor involved in homing of cells to secondary lymphoid organs, and the cellular adhesion molecule, CD62L (or L-selectin) (Campbell *et al.*, 2001). Effector T cells have reduced CCR7 and CD62L expression. This reduces their ability to home to lymph nodes, although they have increased capacity to migrate to inflamed tissues, due to an increase in expression of chemokine receptors such as CCR5 and CCR2. Within the CCR7- CD62L- effector population there is also an enriched CD27- population (Campbell *et al.*, 2001). Central memory T cells express CCR7 and CD62L, and therefore preferentially circulate through the lymphoid tissues (Mackay, 1999; Sallusto *et al.*, 1999). These cells do not have immediate effector function and only secrete IL-2, although they can differentiate into effector cells. Naïve cells are defined by the expression of CD45RA and high

expression of CCR7, CD27 and CD62L. The expression of many of the markers used to define cell populations are largely overlapping, so a better definition of naïve and memory cell phenotype would be desirable.

### 1.2.0 The Leukocyte Common Antigen (CD45).

First described in 1977, the leukocyte common antigen, CD45, is a transmembrane protein tyrosine phosphatase that is known to be essential for T cell signalling. Also referred to as T200, B220 or Ly-5, this high molecular weight glycoprotein is expressed on nucleated haemopoetic cells and can comprise up to 10% of the cell surface (Alexander, 1997).

Both CD45-deficient humans and mice are severely immunodeficient (Kishihara *et al.*, 1993; Tchilian *et al.*, 2001c). Thymocyte development is blocked in CD45-deficient mice, where an inability to positively select T-cells, leads to severely reduced numbers of peripheral T-cells (Byth *et al.*, 1996). The block in antigen receptor signal transduction in CD45 deficient cells is believed to be due to dysregulation of the Src family of tyrosine kinases. Src family members have been shown to be both positively and negatively regulated by CD45 phosphatase activity (Thomas and Brown, 1999; Alexander, 2000; Penninger *et al.*, 2001). Recent evidence also suggests that CD45 phosphatase activity can regulate Janus kinases (JAK) (Irie-Sasaki *et al.*, 2001). JAKs are involved in the activation of type I and type II cytokine receptors, which have a role in the differentiation, proliferation and anti-viral activity of haemopoetic cells

### 1.2.1 The structure of CD45

The gene encoding human CD45, the protein tyrosine phosphatase receptor-type C gene (*PTPRC*), is located on chromosome 1 (1q31-1q32) (Alexander, 1997). There are several other genes of immunological interest close to this region of the chromosome (such as those encoding the Fc IgG1/IgG2A receptors). The *PTPRC* gene is thought to be around 120Kb and contains 34 exons. Although little is known about the regulation of the gene, the highly conserved first intron of the gene has been found to have strong

promotor activity (Timon and Beverley, 2001). Exon 1, encodes a 5' untranslated sequence, and is spliced into two alternatively used exons, 1a and 1b, with exon 1a preferentially expressed. There are three sites for the initiation of transcription, P1a, P1b and P2 (Saga *et al.*, 1987; Hall *et al.*, 1988).

CD45 is a type 1 transmembrane protein, containing a large extracellular domain of 391-552 amino acids (Hall *et al.*, 1988). The amino terminal of this domain contains the variable exons 4, 5 and 6. The CD45 molecule is highly glycosylated, mostly attributed to N-glycosylation on the extracellular domain and O-glycosylation of the variable exons. This glycosylation pattern is not only dependant upon the variable exon usage, but also on the developmental stage, cell type and activation state, suggesting it is functionally important. The extracellular region also contains a cysteine rich domain and three fibronectin type III domains. The fibronectin domains are important for the structural integrity of the molecule as they serve as a platform for N-linked carbohydrate groups. The 700 amino acid cytoplasmic tail, contains two phospho-tyrosine phosphatase domains, the first of which has enzyme activity, whilst the second is thought to be important for protein folding and substrate binding (Alexander, 1997). Molecular modelling also indicates that the juxtamembrane region may form a putative wedge (Bilwes *et al.*, 1996). These structural features are outline in figure 1.5.

Homologues of CD45 have been recognised in a range of species including the chicken, the horned shark (*Heterodontus francisci*) (Okumura *et al.*, 1996), the puffer fish (*Fugu rubripes*) (Diaz del Pozo *et al.*, 2000) and various mammals (Okumura *et al.*, 1996; Ballingall *et al.*, 2001). Interestingly CD45 has recently been described in the Pacific Hagfish (*Eptatretus stoutii*) (Nagata *et al.*, 2002). This would be consistent with the role CD45 in signalling through cytokine receptors, as the jawless vertebrates are believed to lack the adaptive immune system. The cytoplasmic domain of CD45 is highly conserved between species (Okumura *et al.*, 1996) suggesting an essential function for CD45 through divergent evolution of the vertebrate immune system.

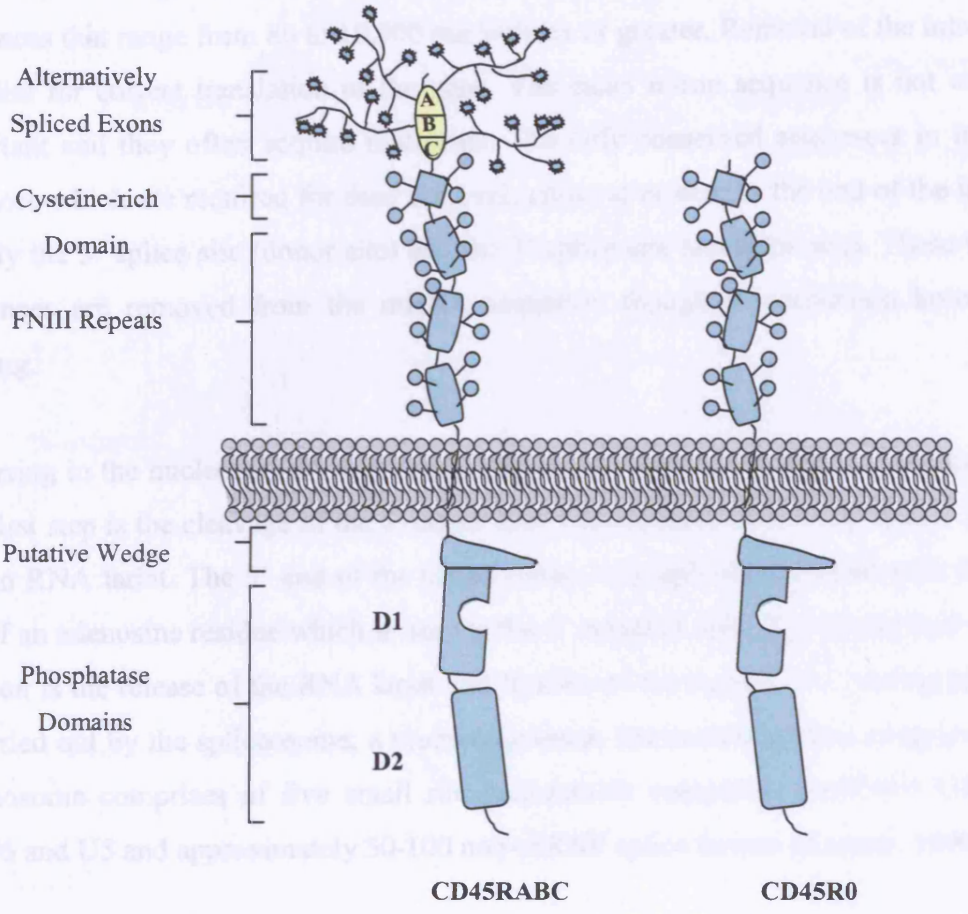



**Figure 1.5 Schematic diagram of the structure of CD45.** CD45 exists as multiple isoforms due to alternative splicing. Figures shows the largest (CD45RABC) and smallest (CD45R0) isoforms (not drawn to scale). The variable exons have multiple O-glycosylation sites. The remaining part of the extracellular domain consists of a cysteine rich region followed by three fibronectin type III repeats, and is heavily N-glycosylated. The large cytoplasmic tail contains two phosphatase domains, D1 and D2, although only D1 has enzymatic activity. Molecular modelling data indicates that the juxtamembrane region may form a structural wedge. (Adapted from Hermiston *et al.*, 2002).

### 1.2.2 Alternative splicing of CD45

#### Splicing mechanisms

Unlike bacterial genes, those of eukaryotic organisms contain introns, non-coding sequences that range from 80 to 10,000 nucleotides or greater. Removal of the introns is essential for correct translation of the gene. The exact intron sequence is not usually important and they often acquire mutations. The only conserved sequences in introns are those which are required for their removal, either at or near to the end of the intron; namely the 5' splice site (donor site) and the 3' splice site (acceptor site). These intron sequences are removed from the mRNA sequence, though a mechanism known as splicing.

Occurring in the nucleolus, RNA splicing is a two step process (outlined in figure 1.6). The first step is the cleavage of the 5' donor site, which results in an exon with a 3' end and an RNA lariat. The 5' end of the intron forms a phosphodiester bond with the 2'-OH of an adenosine residue which is near to the 3' acceptor site. The second step of the reaction is the release of the RNA lariat and ligation of the exons. The splicing process is carried out by the spliceosome, a multicomponent, ribonuclear protein complex. The spliceosome comprises of five small ribonucloprotein complexes (snRNPs) U1, U2, U4/U6 and U5 and approximately 50-100 non-snRNP splice factors (Kramer, 1996).

During the first step of the process the spliceosome is assembled. The U1 snRNP binds to the 5' splice site, guided by a nucleotide sequence in the U1 RNA that forms complementary base pairs to the nine-nucleotide splice site consensus sequence. Branch point A is then recognised by the branch point binding protein (BBP), which is then replaced by U2. The U2 snRNP binding also requires an auxiliary factor, U2AF, which binds to polypyrimidine tracts near to the 3' splice site by RNA recognition motifs (RRM) and arginine-serine-rich (RS)-domains. This complex then binds U4-U6-U5, bringing the exons together. U4 snRNP then dissociates from U6. U1 becomes destabilised at the 5' splice site, and is replaced by U6 as it forms a helix with U2, moving the branch point close to the 5' splice site. The exon is then excised at the 5' splice site by a transesterification reaction, and the intron forms a lariat, where the 5' end interacts with an adenosine at the branch point. The free 3'-OH at the end of the 5'

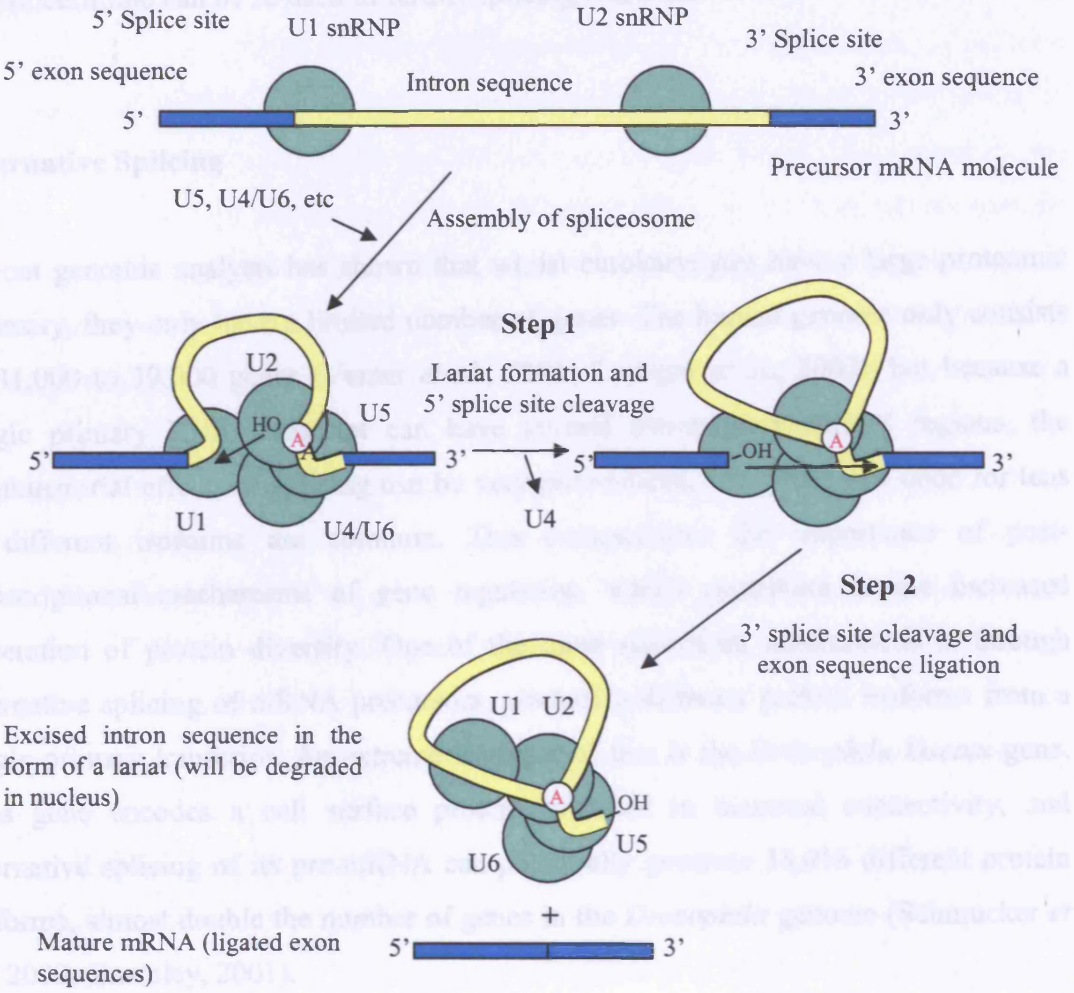



**Figure 1.6 Schematic diagram of the RNA splicing mechanism.** RNA splicing is catalysed by a spliceosome formed from the assembly of U1, U2, U5 and U4/U6 snRNPs (depicted as green circles) and other molecules (not shown). After spliceosome assembly the reaction occurs in two steps, firstly, lariat formation and cleavage of the 5' splice site, and secondly 3' splice site cleavage and ligation of the exon sequence. The exon sequences are therefore jointed together and intron sequence is released as a lariat and will subsequently degrade.

exon interacts with the U5 snRNP and is positioned close to the 3' splice site. After some conformational changes, the second step of the splicing process results in the joining of the two exons. After ligation the spliceosome complex dissociates, the snRNPs detach from the lariat RNA which is degraded by RNAases. The components of the spliceosome can be re-used in further splicing reactions.

**Alternative Splicing**

Recent genomic analysis has shown that whilst eurokaryoytes have a large proteomic diversity, they only have a limited number of genes. The human genome only consists of 31,000 to 39,000 genes (Venter *et al.*, 2001; Cartegni *et al.*, 2002), but because a single primary RNA transcript can have several alternatively spliced regions, the combinatorial effects of splicing can be very pronounced, and genes that code for tens of different isoforms are common. This demonstrates the importance of post-transcriptional mechanisms of gene regulation, which contribute to the increased generation of protein diversity. One of the most significant mechanisms is through alternative splicing of mRNA precursors, producing different protein isoforms from a single primary transcript. An extreme example of this is the *Drosophila Dscam* gene. This gene encodes a cell surface protein involved in neuronal connectivity, and alternative splicing of its pre-mRNA can potentially generate 38,016 different protein isoforms, almost double the number of genes in the *Drosophila* genome (Schmucker *et al.*, 2000; Graveley, 2001).

The mechanisms of splice site selection are closely connected in both constitutive and alternative splicing, as many of the components of the essential machinery for constitutive splicing also have a role in the regulation of alternative splicing. Compared to constitutive exons, alternative exons are often found to have suboptimal splice sites and / or length. Splicing of alternative exons can be modulated by trans-acting factors, which recognise an arrangement of positive (splicing enhancers) and / or negative (splice silencers) or *cis*-acting sequence elements (either exonic of intronic). Differences in the amounts or activities of a variety of splicing factors during development or in different tissues can cause differential patterns of splicing.

Alternative splicing can occur as a result of a variety of factors, such as alternative promoter usage (Nabeshima *et al.*, 1984), alternative poly(A) site usage (Early *et al.*, 1980) or transcriptional factors, such as the protein-recruiting and elongating properties of the transcription machinery. Some of the major trans-acting splice factors known to have a role in alternative splicing include the SR protein family, the polypyrimidine tract binding protein (PTB), and the CELF protein family. The CELF family of proteins (CUG-BP and ETR3-like factors) are involved in cell-specific and developmentally regulated alternative splicing in the striated muscle and brain. They promote the inclusion of exon 5 of the cardiac troponin-T gene (cTNT) by binding muscle specific enhancers. PTB, also known as hnRNP 1, is an RNA binding protein which recognises polypyrimidine tracts preceding the 3' splice site. It has a role as a negative regulator of splicing, mainly by competing with U2AF in binding to the polyprimidine tract.

A group of highly conserved proteins, required for constitutive splicing, the SR family proteins, also have a role in alternative splicing. These proteins have a modular structure, consisting of an RNA-recognition motif (RRM) and a C-terminal domain, the RS domain, which is rich in alternating serine and arginine residues. RNA binding specificity is determined by the RRM's, whilst the RS domain mediates protein-protein interactions. These interactions are thought to be necessary for the recruitment of the splicing apparatus and for splice site pairing (Wu and Maniatis, 1993; Tacke and Manley, 1999). The SR related protein (SRrps) are another class of RS domain containing proteins, which may contain RRM's and are involved in splicing. These proteins include U1-70K and U2AF, as well as regulators of alternative splicing, such as Tra and Tra2. Both the SR family and SR-related proteins function by recognising exonic splicing enhancers (ESEs) and leading to the activation of suboptimal adjacent 3' splice sites (Blencowe, 2000).

Around 15% of the mutations which cause genetic disease affect pre-mRNA splicing. Hereditary disease is frequently caused by mutations in no-coding regions, such as those affecting 5' and 3' splice sites, branch sites or polyadenylations signals (Krawczak *et al.*, 1992). Analysis of 50 single base substitutions causing exon skipping in human genes (including missense, nonsense and translationally silent mutations) showed that at least one of the target motifs for the SR proteins SF2/ASF, SRp40, SRp55 and SC35, found in ESEs were disrupted (Liu *et al.*, 2001a). This is interesting as it would suggest

that one of the main causes of exon skipping is single base alterations in ESEs, the splicing enhancer sequences.

**CD45 alternative splicing**

Alternative splicing of exons 4, 5 and 6 (also referred to as exons A, B and C) at the N-terminus of the extracellular domain of CD45, could potentially generate 8 different isoforms (figure 1.7) although only 5 of these have been identified at the protein level in humans (Streuli *et al.*, 1987). The highest molecular weight isoform, CD45RABC contains all three variable exons (4, 5 and 6). In the lowest molecular weight null isoform, CD45R0 all three exons have been spliced out (Penninger *et al.*, 2001). In murine T cells and cell lines, there is some evidence that exons 7, 8 and 10 can also be alternatively spliced (Chang *et al.*, 1991; Virts *et al.*, 1998). So far this has only been demonstrated at the mRNA level and protein expression has not been established. A smaller CD45 isoform, CD45iota, has been described in murine Th1 cells, which has exons, 4, 5, 6 and 7 spliced out (Tsujikawa *et al.*, 2000). CD45iota mRNA has been found present in the foetal thymus and splenic T cells of mice and in murine Th1 but not Th2 clones. The observed difference in the expression pattern of the CD45iota isoform could be a method of modulating the T helper cell activation threshold, selectively controlling lymphocytes and modulating cytokine signalling. However, this isoform has not been identified in humans so is very unlikely to have a role in human T cells.

The expression of CD45 isoforms is highly regulated and dependent upon the activation and differentiation states of haemopoietic cells (Akbar *et al.*, 1988; Mackay, 1990; Novak *et al.*, 1994). The cell surface expression pattern of the major isforms, in different cell types, in humans is outlined in figure 1.8. In some species, such as humans and mice, it has been used to distinguish between different functional subsets of lymphocytes. B cells express the high molecular weight (220kD) isoform CD45RABC (also called B220), whilst immature CD4+CD8+ T cells express mainly the low molecular weight isoforms. Mature CD4+ and CD8+ thymocytes and peripheral T cells can express multiple isoforms. Additionally, isoform expression may alter during T cell activation. Natural Killer (NK) cells are also known to express both the high and low molecular weight isoforms of CD45 (Mysliwski *et al.*, 2001), although little is known

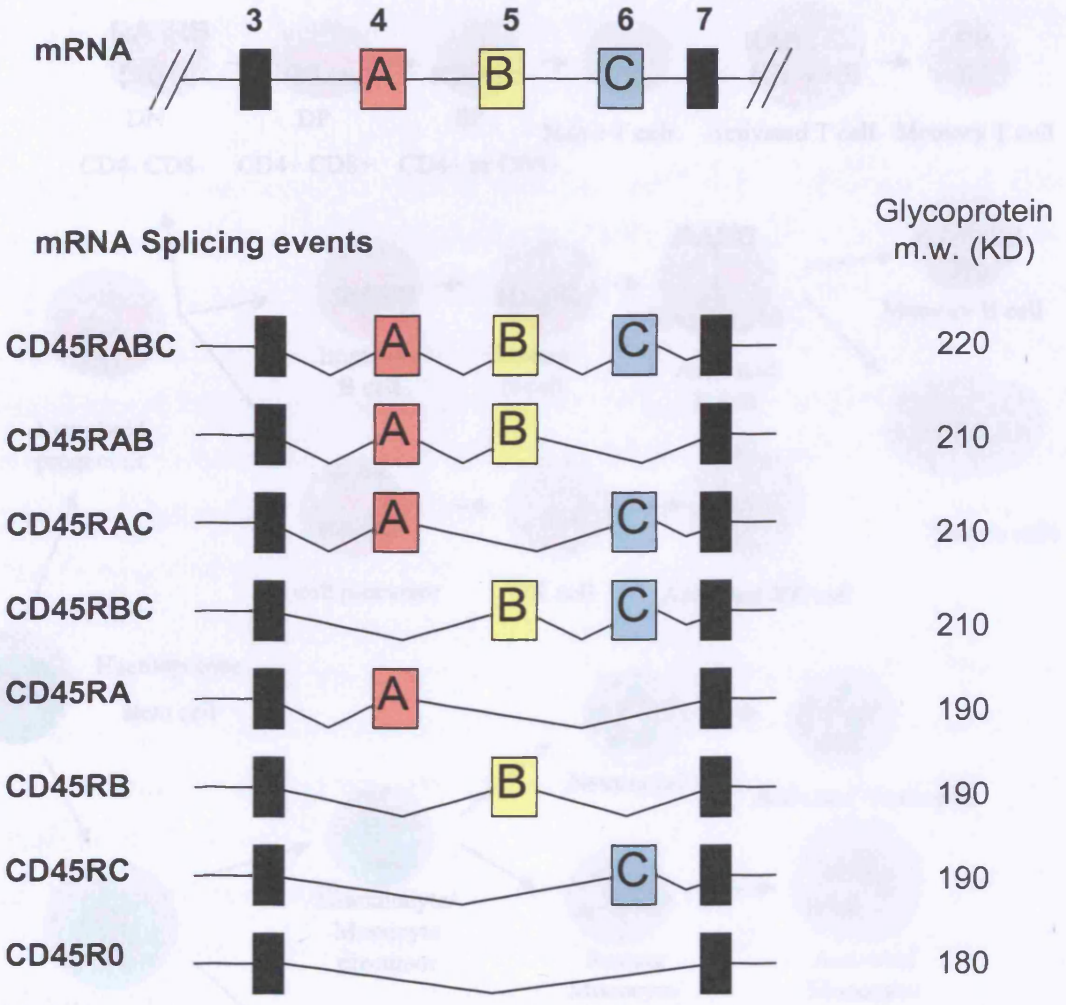



**Figure 1.7 Schematic representation of the CD45 alternatively spliced region and the eight possible mRNA transcripts generated by alternative splicing.** Exons are represented as boxes and introns represented as lines (not to scale). The molecular weight of the various proteins isoforms are given in kilodaltons.

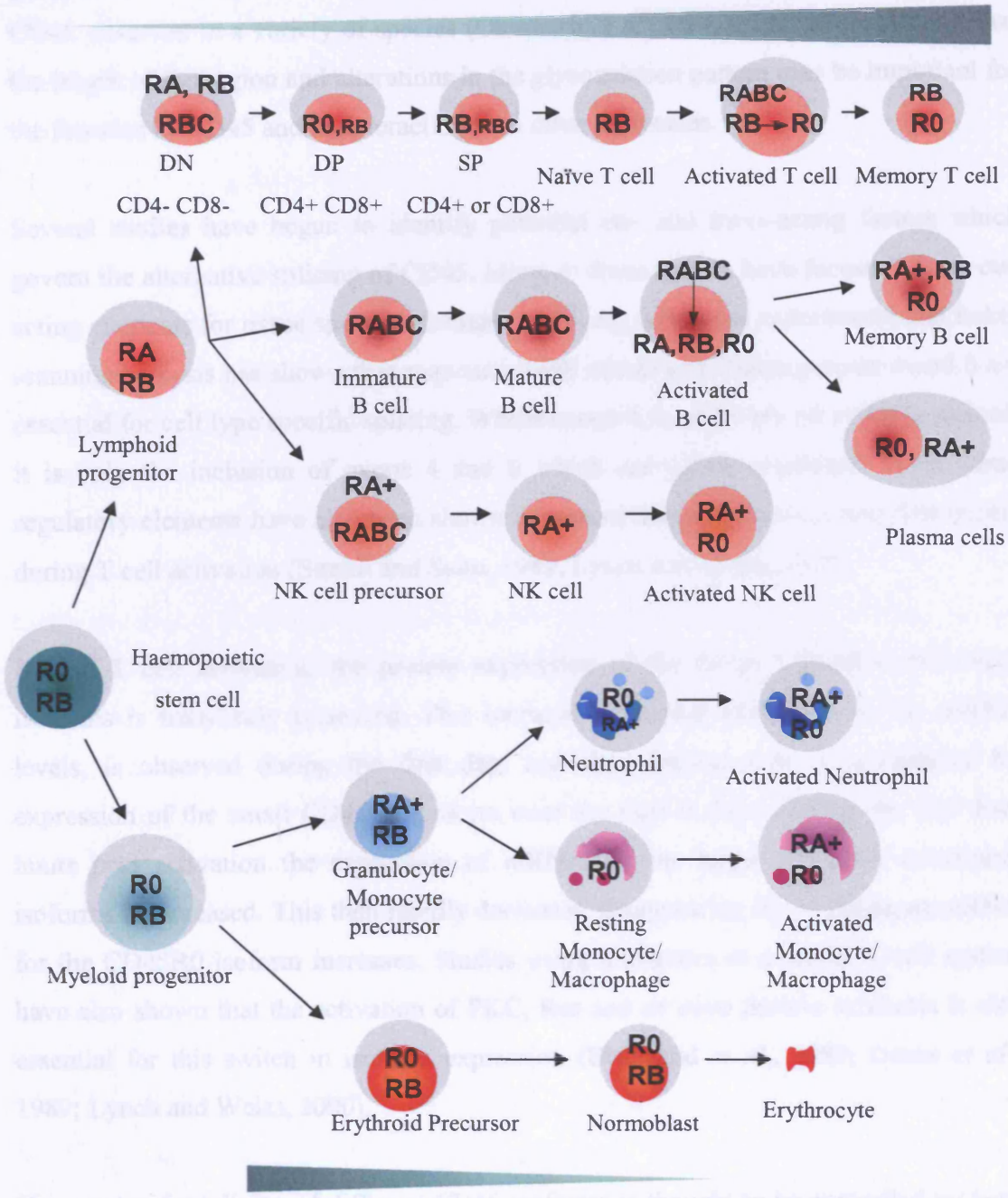



**Figure 1.8 Cell surface Expression patterns of CD45 isoforms.** Schematic representation, only predominant isoforms depicted. Less abundant isoforms are denoted in smaller font. Triangles represent increasing expression of the protein during development of T, B and myeloid cells and decreasing expression during erythroid differentiation, with the exception of plasma cells, which have decreased total CD45 expression (adapted from Hermiston *et al.*, 2002).

about the function of CD45 in NK cell responses. However, the exact function of the different CD45 isoforms remains obscure. The variable exons are heavily O-glycosylated, and alternative splicing of this region appears to be a conserved feature of CD45 observed in a variety of species (Okumura *et al.*, 1996). This could suggest that the length of the region and alterations in the glycosylation pattern may be important for the function of CD45 and its interaction with other molecules.

Several studies have begun to identify potential *cis*- and *trans*-acting factors which govern the alternative splicing of CD45. Many of these studies have focused on the *cis*-acting elements for tissue specific alternative splicing. Minigene experiments and linker scanning analysis has shown that sequences both within and flanking exons 4 and 6 are essential for cell type specific splicing. Whilst exons 4, 5, and 6 are all variably spliced, it is only the inclusion of exons 4 and 6 which are tightly regulated. These same regulatory elements have also been shown to control the isoform switching that occurs during T cell activation (Streuli and Saito, 1989; Lynch and Weiss, 2001).

During T cell activation, the protein expression of the larger CD45RA containing isoforms is transiently increased. This increase, reflecting alterations in the mRNA levels, is observed during the first day, and then decreases as it is replaced by expression of the small CD45R0 isoform over the next 3 days. During the first four hours post activation the expression of mRNA for the larger CD45RA containing isoforms is increased. This then rapidly decreases, disappearing by 24 hours, as mRNA for the CD45R0 isoform increases. Studies using minigenes in a model T cell system have also shown that the activation of PKC, Ras and *de novo* protein synthesis is also essential for this switch in isoform expression (Birkeland *et al.*, 1989; Deans *et al.*, 1989; Lynch and Weiss, 2000).

Tissue specific splicing of different CD45 isoforms is thought to be controlled by both positively and negatively regulatory *trans*-acting factors which allow the exons to be spliced out (Rothstein *et al.*, 1991 1994; Trowbridge and Thomas, 1994). One candidate for mediating tissue-specific splicing of CD45 is the SR proteins previously mentioned. These are regulated by phosphorylation and expressed in a tissue specific manner. In model systems expressing CD45 minigenes and over expressing various SR proteins, it has been shown that SRp20 and 9G8 can facilitate exon inclusion, whilst SF2/ASF,

SC35, SRp30c, SRp40 and SRp70 promote exon exclusion (Sarkissian *et al.*, 1996; Lemaire *et al.*, 1999; ten Dam *et al.*, 2000).

### 1.2.3 Role of CD45 in development and signalling

Initial studies using CD45-deficient cell lines identified CD45 as an obligate positive regulator of antigen receptor signalling (Pingel and Thomas, 1989; Koretzky *et al.*, 1990; Hovis *et al.*, 1993; Volarevic *et al.*, 1993; Desai *et al.*, 1994). It was subsequently observed that both CD45-deficient humans and mice are severely immunodeficient (Kishihara *et al.*, 1993; Byth *et al.*, 1996; Mee *et al.*, 1999; Kung *et al.*, 2000; Tchilian *et al.*, 2001c). CD45-deficient mice have been made by several different groups, by targeting exons 6, 9 or 12. All of these mice have profound defects in thymic development, due to increased apoptosis and dysfunctional signalling through both the pre-TCR and TCR complexes. These mice were found to have reduced numbers of both single and double positive thymocytes. Despite proceeding normally until the final stage in development, B cell maturation is also affected in CD45-/- mice. There is a two-fold increase in the total number of B cells, but there are decreased numbers of mature (IgM low, IgD high) cells in both the spleen and periphery; the remaining cells are mainly immature IgM high, IgD low cells in the spleen (Kishihara *et al.*, 1993; Benatar *et al.*, 1996; Byth *et al.*, 1996). CD45 is an essential modulator of signalling threshold in B cells. Although the function of CD45 is less well defined in B cells, it is thought to be similar for BCR signalling as TCR signalling, regulating phosphorylation of Src family kinases.

One of the earliest events in TCR signalling is initiated by Lck and Fyn, which are both Src family kinases (SFK). P56Lck is constitutively associated with the co-receptor molecules, CD4 and CD8. Upon receptor clustering, p59fyn associates with the cytoplasmic domains of the $\zeta$ and CD3$\varepsilon$ chains. Therefore antigen recognition allows both Fyn and Lck to phosphorylate specific ITAMS on the accessory chains of the TCR complex. The enzyme activity of the SFKs is regulated by phosphorylation of the regulatory tyrosine residues at amino acids 394 and 505 in the kinase domain and carboxy-terminal region. Phosphorylation of tyrosine 394 in the kinase domain is

activatory, whilst that of tyrosine 505 in the carboxy-terminal domain is inhibitory. Upon antigen-specific activation, the inhibitory tyrosine (505) is dephosphorylated, allowing autophosphorylation of the activatory tyrosine (394), and restoring catalytic activity.

However, even after phosphorylation of the activatory tyrosine the SFKs can still be kept inactive by Csk (C-terminal SRC kinase), a protein tyrosine kinase. In resting cells, Csk activity is constitutive, so the Src proteins are usually inactive. CD45 counteracts this effect as it can remove phosphates from phosphotyrosine, particularly from the inhibitory tyrosine reside of SFKs. Signalling activity of the SFKs in response to receptor aggregation is therefore determined by the balance between Csk, which prevents their activation, and CD45 which restores their potential to be activated.

This idea is supported by the fact that in most CD45-deficient cell lines and CD45-/- thymocytes, both lyn and fyn are hyperphosphorylated at their negative regulatory tyrosine, and the TCR is completely uncoupled from intracellular signals. In addition to this it has been demonstrated that thymic development can be rescued in CD45-/- mice by expression of a constructively active Lck Y505F mutant. This shows that this negatively regulatory tyrosine is a physiologically relevant substrate for CD45 (Pingel and Thomas, 1989; Koretzky *et al.*, 1990; Cahir McFarland *et al.*, 1993; Sieh *et al.*, 1993; Stone *et al.*, 1997; Pingel *et al.*, 1999; Seavitt *et al.*, 1999). However, it has also been observed that despite hyperphosphorylation of the negatively regulatory tyrosine, cellular SFK activity is actually increased in CD45-/- thymocytes and some CD45 deficient cells lines (Biffen *et al.*, 1994; Ashwell and D'Oro, 1999; D'Oro and Ashwell, 1999). This is thought to be due to an increased phosphorylation of the autocatalytic site in these cells, suggesting that this autocatalytic activatory tyrosine could also function as a CD45 substrate.

Therefore, CD45 can modulate the signal transduction thresholds by functioning not only as a positive, but also as a negative regulator of SFKs. It is plausible that the use of different regulatory mechanisms is cell state dependant, corresponding with accessibility of substrate. In resting T cells, CD45 dephosphorylates the negative regulatory tyrosine and to some extent the autocatalytic activatory tyrosine, resulting in signal competent Lck. TCR clustering during antigen recognition may functionally

segregate CD45 from its substrate by exclusion from lipid rafts and the immunological synapse. This will lead to sustained Lck activity whilst the signal transduction cascades are being initiated (Dustin, 2002).

In T cells p59fyn is associated with the T cell receptor. It has two isoforms, p59fynT and p59fynB, generated by alternative splicing of one exon. The p59fynT isoform is present in lymphocytes. As with Lck, fyn has two tyrosine phosphorylation sites, which are regulated in a similar manner. CD45 dephosphorylates the negative regulatory residue (position 528). Subsequent autophosphorylation of the tyrosine residue (417) located within the catalytic domain results in PTK activity (Cooke and Perlmutter, 1989; Koch *et al.*, 1991; Shiroo *et al.*, 1992).

More recently it has been suggested that CD45 may also have a role in negatively regulating cytokine and interferon receptor activation by dephosphorylating Janus Kinases (JAKs) (Irie-Sasaki *et al.*, 2001; Penninger *et al.*, 2001; Yamada *et al.*, 2002). JAKs are positive regulators of cytokine signalling, which function by phosphorylating the signal transducers and activators of transcription (STATs) family of transcription factors. Phosphorylated STATs translocate to the nucleus where they regulate the expression of genes involved in chemokine and cytokine responses.

It is not clear if different CD45 isoforms have equal roles in the regulation of T cell signalling. *In vitro*, different CD45 isoforms have been shown to have the same phosphatase activity and the cytoplasmic tail is sufficient to restore TCR signalling (Trowbridge, 1991; Hovis *et al.*, 1993; Volarevic *et al.*, 1993; Desai *et al.*, 1994). But, further studies with CD45-deficient cell lines or transgenic mice expressing single CD45 isoforms on a knockout background have yielded inconsistent results, with the expression of different isoforms having different effects on signalling. (Chui *et al.*, 1994; Novak *et al.*, ; Leitenberg *et al.*, 1996; Onodera *et al.*, 1996; Kozieradzki *et al.*, 1997; Dornan *et al.*, 2002). The interpretation of these data is difficult, as isoform expression equivalent to physiological levels has not been attained in many studies, meaning that the effects of isoform expression are obscured by the effects of altered total CD45 expression.

### 1.2.4 Mechanisms for CD45 regulation

CD45 has an essential role in T and B cell signalling, so to fully understand the regulation of the immune response it is also necessary to understand the regulation of this highly conserved molecule. Several methods of regulation have been proposed including, ligand binding, dimerisation, localisation (both in membrane structures and accessibility to substrates), interactions with other protein and the action of intracellular inhibitors. It is likely that many if not all of these factors have some role in CD45 regulation.

The extracellular domain of CD45 has an overall rod like structure, which projects from the cell, but can also bend and fold laterally. This structure allows the possibility, of both *cis* and *trans*, ligand interactions. The first possible ligand to be suggested for CD45 was the B cell activation and differentiation marker CD22 (Stamenkovic *et al.*, 1991). It has subsequently been demonstrated that the lectin CD22, interacts with a variety of glycoproteins with N-linked sialic acids, and is therefore not CD45 specific (Sgroi *et al.*, 1993).

Two low molecular weight isoforms of CD45 have been shown to interact in a carbohydrate-dependant manner with the cysteine rich domain of the mannose receptor (Martinez-Pomares *et al.*, 1999). It has further been proposed that the soluble form of the mannose receptor can interact with CD45 and other ligands present in the secondary lymphoid organs. This may suggest a role for CD45 in mediating the delivery of antigen to follicular areas (Martinez-Pomares and Gordon, 1999a). The serum mannose binding protein (S-MBP) has been found to bind to CD45R0 on immature thymocytes with the CD4+CD8+CD3$^{low}$ phenotype. Ligands for S-MBP decrease on thymocyte maturation, suggesting that the oligosaccharide portion of CD45 on immature thymocytes may be associated with maturation, development or selection events (Uemura *et al.*, 1996).

Another lectin that has been proposed to bind with CD45 is galectin–1 (Perillo *et al.*, 1995; Walzel *et al.*, 1999; Symons *et al.*, 2000). Galectin-1, expressed by stromal cells in the thymus and lymph nodes, is specific for β-galactosides, and may be involved in the regulation of apoptosis during T cell development and maturation. Galectin-1 and

galectin-3 can associate with the T-cell antigen receptor complex and co-receptor molecules, modulating TCR mediated signalling. As galectin is involvedin the organisation of cell surface glycoproteins, it has been speculated that the function of CD45 may be regulated by the interaction of CD45 isoforms with a galectin lattice on the cell surface (Demetriou *et al.*, 2001).

Most of the ligands investigated so far have been shown to bind non-specifically to T cell glycoproteins, and there is little evidence that they can actually modulate CD45 phosphatase activity. The extracellular domain of CD45 may participate in *cis* interactions with molecules on the surface of the same cell. Various CD45 isoforms have been shown to interact with different molecules, including Thy1, CD2, LFA1, CD4 and the TCR complex (Novak *et al.*, 1994). Such interactions may alter access to substrate or phosphatase activity. As CD45 is one of the most abundant cell surface proteins it is possible that these molecules are associated by random cross-linking, and expression of multiple isoforms on single cell types makes any associations hard to establish. One interaction which has been established is between CD45 and the lymphocyte phosphatase-associated protein (LPAP) although the exact function of this molecule is not clearly understood (Volarevic *et al.*, 1993; Bruyns *et al.*, 1995). Co-capping and co-immunoprecipitation experiments and FRET analysis have all shown a preferential association between CD45R0 and CD4/CD8 and the TCR (Dianzani *et al.*, 1990; Leitenberg *et al.*, 1996; Leitenberg *et al.*, 1999; Dornan *et al.*, 2002). This may account for the more efficient TCR signalling observed in CD45R0+ cells.

The description of homodimeric forms of CD45 has led to the suggestion that homodimerisation of CD45 may lead to inhibition of its tyrosine phosphatase activity (Takeda *et al.*, 1992; Majeti *et al.*, 2000; Xu and Weiss, 2002). Thus, Xu and Weiss (2002) proposed a model of CD45 regulation where monomeric and dimeric isoforms exist in equilibrium on the cell surface. This equilibrium determines the overall CD45 activity of the cell, which is regulated by isoform expression. CD45R0 readily forms homodimers as it does not have the negative charges and bulky O-linked glycoconjugates of the CD45RA isoform, and these homodimers can down regulate TCR activity. In addition to isoform switching, during cell lineage differentiation and lymphocyte activation, total CD45 expression can be up-regulated. This increased expression may alter the monomer / homodimer equilibrium, so may also have a role in

facilitating dimerisation. However, whilst plausible, dimerisation cannot be the only mechanism of regulation, as it does not explain how memory cells expressing CD45R0 can respond more rapidly than naïve CD45RA cells in the primary response.

The localisation of the CD45 molecule with respect to lipid rafts and the immunological synapse is an area of some debate. In unstimulated Jurkat cells CD45 is absent from membrane lipid rafts. Engagement of the TCR promotes lipid raft aggregation, which subsequently promotes the localisation of raft associated proteins, the TCR complex, Lck and LAT, but excludes CD45 (Rodgers and Rose, 1996; Xavier and Seed, 1999). However other studies using human leukaemia cell lines and mouse thymocytes have shown some association of CD45 with parts of the lipid raft (Parolini *et al.*, 1996; Ilangumaran *et al.*, 1999). This discrepancy may be due to differences in the techniques used and definition of raft structure. Irles *et al.*, (2003) have further described a role for the CD45 ectodomain in the control of Lck activity through its interaction with glycosphingolipid-enriched membranes (GEMs). The association of CD45 to the immunological symapse (IS) / supramolecular activation cluster (SMAC) is also of some debate. The CD45 molecule is large compared to the other molecules involved in antigen-specific recognition, and therefore would be predicted to be excluded from the IS/SMAC on the basis of size. Whilst some studies support this notion, others have shown the presence of CD45 at the site of TCR-APC interaction or at least an ability to migrate back into the IS/SMAC, but to a distinct region from that of the TCR (Shaw and Dustin, 1997; Sperling *et al.*, 1998; Johnson *et al.*, 2000; Leupin *et al.*, 2000; Freiberg *et al.*, 2002). Again this discrepancy is probably due to different techniques and cells lines used, but is an interesting area for further study.

### 1.3.0 CD45 Polymorphisms and disease association

### 1.3.1 CD45 and disease

Abnormal CD45 expression has been associated with a number of diseases. A homozygous 6 base pair deletion in exon 11 of CD45, observed in a Kurdish infant, resulted in a lack of surface CD45 expression and severe combined immunodeficiency (SCID) (Tchilian *et al.*, 2001c). Lack of CD45 surface expression has been linked to two separate genetic abnormalities in another SCID patient (Kung *et al.*, 2000). This shows that CD45 has an important immune function in humans, and that lack of CD45 expression can be a cause of SCID. Similarly a variety of mouse models have been described which suggest that modulation of CD45 may affect autoimmunity, cancer and transplantation (Penninger *et al.*, 2001), making it a strong potential candidate for immunotherapy (Basadonna *et al.*, 1998).

Because of the correlation between isoform expression and activation state of T cells, a number of studies have investigated alterations in isoform expression during disease. Associations have been reported in a number of diseases, including SLE (systemic lupus erythematosus), rheumatoid arthritis and HIV (Penninger *et al.*, 2001). Alterations in CD45 function have also been implicated in hematologic malignancies and Alzheimer's disease. In a transgenic mouse model of Alzheimer's disease, modulation of CD45 isoforms can activate microglia. Additionally in Alzheimer's patients, CD45 expression was found to be up-regulated on microglial cells and cross-linking CD45 suppressed CD40 ligand-induced and β-amyloid peptide-induced microglia activation. However, whether this increased CD45 expression represents a cause or effect of the disease remains unclear (Basadonna *et al.*, 1998; Tan *et al.*, 2000a; Tan *et al.*, 2000b; Penninger *et al.*, 2001). Some studies of SLE patients have shown decreased CD45 expression and / or phosphatase activity, suggesting that alteration of CD45 activity may have a role in autoimmune disease. It is plausible that altering the balance between positive and negative regulation of tyrosine phosphorylation could contribute to the development of autoimmune disease. However, it is also possible that the observed CD45 down-regulation is an effect of the disease or of the immune system's attempt to control it (Takeuchi *et al.*, 1997; Blasini *et al.*, 1998a; Blasini *et al.*, 1998b).

The exact function of each of the CD45 isoforms remains elusive, but expression of the different isoforms is known to alter TCR-mediated signal transduction (Leitenberg *et al.*, 1999). Previously in studies with transgenic mice the expression of only the high molecular weight isoform has been shown to compromise immune function (Kozieradzki *et al.*, 1997). These mice could not generate cytotoxic T cell responses or neutralising antibodies after a viral infection. However, the transgene in these mice was under the control of an Lck promoter, which confers thymic specific expression. This resulted in a 10 fold difference in the expression of both the CD45RABC and CD45R0 isoforms in the periphery, so no conclusions could be made regarding cells in the peripheral lymph nodes. However more recent studies with transgenic mice expressing single CD45 isoforms (discussed further in Chapter 7), have shown that expression of either high or low molecular weight isoforms can restore immune function, but it is the level of expression which is important (Ogilvy *et al.*, 2003; Tchilian *et al.*, 2004). The role of CD45 in the dephosphorylation of the Janus Kinases also indicates a function for CD45 in the control of cell proliferation, differentiation and cytokine mediated anti-viral activities. The regulation of cytokine signalling in particular may be an important factor in disease.

### 1.3.2 The C77G polymorphism

Abnormalities in the splicing of CD45 have been recognised in humans. In 1990 Schwinzer and Wonigeit described a group of healthy individuals with variant CD45 expression. T cells from these individuals constitutively express CD45RA, failing to switch to single CD45R0 expression even after mitogenic stimulation. This splice defect was later identified as being caused by a single point mutation in exon 4 (also known as exon A) of the *PTPRC* gene. This Cytosine to Guanine transversion mutation at nucleotide position 77 of exon 4 (C77G) has been shown to prevent N-terminal splicing of the gene (Thude *et al.*, 1995; Tchilian *et al.*, 2001b). The C77G mutation does not change the amino acid sequence, but disrupts a strong exonic silencer, preventing splicing (Thude *et al.*, 1995; Zilch *et al.*, 1998; Lynch and Weiss, 2001). In C77G heterozygous individuals, activated or memory lymphocytes express both the high and

low molecular weight isoforms, rather than only low molecular weight isoforms, which are normally observed.

The frequency of the C77G polymorphism amongst the healthy population is variable but usually low (Table 1.1). In the UK, the frequency of the allele was found to be 0.85%, as opposed to 0.16% in Germany and Italy, whilst in North America and Sweden the frequencies were found to be 1.4 and 1.8% respectively. The polymorphism was found to be absent in African populations, whilst in central Asians, it was similar to that in Northern Europeans (Table 1.1). The highest frequency of the C77G polymorphism (6.7%) was found in the Pamiris, a highly endogamous population, who live in the remote mountain valleys of Gorno Badakshan (Tajikistan) (Tchilian and Beverley, 2002) The C77G polymorphism was found to be extremely rare or absent from the Japanese population (Stanton *et al.*, 2003). The reason for this frequency variation between different populations is unclear, but further analysis may reveal more about the role of the mutation, giving an indication of the selective pressures that may be involved in its distribution. Alternatively the variant may have originated in Europe and spread by subsequent migration to Asia (Wells *et al.*, 2001).

### 1.3.3 C77G and disease association

Three different case control studies in Germany (Jacobsen *et al.*, 2000) and one in Italy (Ballerini *et al.*, 2002) have suggested an association of the C77G with multiple sclerosis. However, similar studies in the US, Sweden,(Vorechovsky *et al.*, 2001) Italy and another in Germany (Miterski *et al.*, 2002) failed to show any association. An increased frequency of the variant C77G allele has been reported in HIV-1 positive individuals in the UK (Tchilian *et al.*, 2001a). Two patients with abnormal CD45 splicing and displaying the variant C77G allele, have been described with haemophagocytic lymphohistiocytsis and erythrocytic hemophagocytosis (HLH syndrome) (McCormick *et al.*, 2001). Three C77G variant individuals have been described with LCH (Langerhans cell histiocytosis), a rare disease which can be invoked by environmental triggers, such as viral infections (Willman *et al.*, 1994; Boxall *et al.*, 2004). A patient with common variable immunodeficiency (CVID) and a history of prolonged excretion of poliovirus has also been found to have the variant

| Polymorphism | Population | Allele Frequency % | Reference |
|---|---|---|---|
| Exon 4 C77G | UK (236, 181) | 0.85-1.1 | (Tchilian et al. 2002; Stanton et al. 2003) |
| | Orkney (72) | 3.5 | (Stanton et al. 2003) |
| | Sweden (1044) | 1.4 | (Vorechovsky et al. 2001) |
| | Germany (303, 377) | 0-1.4 | (Jacobsen et al.2000; Miterski et al.2002) |
| | Italy (222, 529) | 0-0.9 | (Ballerini et al. 2002; Gomez-Lira et al.2003) |
| | Central Asia (279, 139) | 1.6-2.1 | (Tchilian et al.2002; Stanton et al. 2003) |
| | Pamiris (75) | 6.7 | (Tchilian et al. 2002) |
| | South East Asia (143) | 0.0 | (Tchilian et al. 2002) |
| | Japan (175) | 0.0 | (Stanton et al. 2003) |
| | Korea (48) | 0.0 | (Stanton et al. 2003) |
| | USA (244) | 1.8 | (Barcellos et al. 2001) |
| | Uganda (93, 209) | 0.0 | (Tchilian et al.2002; Stanton et al. 2003) |
| Exon 4 A54G | Uganda (109) | 0.45 | (Stanton et al. 2004) |
| | UK & Orkney (181, 72) | 0.0 | (Stanton et al. 2004) |
| | Japan (175) | 0.0 | (Stanton et al. 2004) |
| | Korea (48) | 0.0 | (Stanton et al. 2004) |
| | Malawi (40) | 0.0 | (Stanton et al. 2004) |
| Exon 4 C59A | Germany (206) | 0.0 | (Jacobsen et al. 2002) |
| Exon 4 C77T | Italy (204) | 0.0 | (Gomez-Lira et al. 2003) |
| Exon 5 G69C | Italy (204) | 0.5 | (Gomez-Lira et al. 2003) |
| Exon 6 T127A | Italy (204) | 0.3 | (Gomez-Lira et al. 2003) |
| Exon 6 A138G | UK (181) | 0.3 | (Stanton et al. 2003) |
| | Orkney (72) | 0.7 | (Stanton et al. 2003) |
| | Italy (204) | 1.0 | (Gomez-Lira et al. 2003) |
| | Tatar (65) | 4.6 | (Stanton et al. 2003) |
| | Japan (175) | 23.7 | (Stanton et al. 2003) |
| | Korea (48) | 7.3 | (Stanton et al. 2003) |
| | Uganda (209) | 0.0 | (Stanton et al. 2003) |

**Table 1.1 Frequency of CD45 variant alleles in healthy controls from different populations in published studies.** Number of individuals is indicated in brackets.

C77G polymorphism and abnormal CD45 splicing (E.Tchilian, personal communication). Therefore it may be speculated that abnormal CD45 splicing may be associated with impairment in anti-viral responses or clearance of the virus. Further studies have suggested an association between the C77G polymorphism and autoimmune diseases such as systemic sclerosis, SSc (Schwinzer *et al.*, 2003) and Autoimmune hepatitis, AIH (Vogel *et al.*, 2003).

We have also analysed the frequency of the C77G allele in a cohort of patients with hepatitis C, from the UK (E. Tchilian, Personal communication). Of 388 hepatitis C patients, fifteen were found to be heterozygous for the C77G polymorphism, an allele frequency of 1.9%. This is twice the frequency observed in the uninfected control population (0.95%) where only 8 out of 417 individuals carried the C77G polymorphism. This could suggest that the C77G polymorphism is associated with poor prognosis on viral infection, at least to the hepatitis C virus (HCV). To determine if there is an association of the variant C77G allele and disease outcome, the frequency of the polymorphism between hepatitis C chronic patients, who are unable to clear the virus (HCV RNA positive) and individuals who resolved HCV infection and became HCV RNA negative was investigated. Remarkably, of the 256 chronic carriers (HCV RNA positive), 14 were heterozygous for the C77G polymorphism (allele frequency of 2.7%) whilst only one C77G heterozygous individual was identified in 132 HCV RNA negative individuals (allele frequency 0.3%). This is statistically significant ($p=0.02$) and suggests a link between the presence of the C77G polymorphism and the outcome of the hepatitis C infection, with an inability to clear the virus.

### 1.3.4 The A138G polymorphism

We have recently described a novel polymorphism, A138G (Thr47Ala) in Exon 6 (Gomez-Lira *et al.*, 2003; Stanton *et al.*, 2003). This polymorphism has been found to be prevalent in Japanese (23.7%) and Korean (7.3%) populations, with 5.1% of the Japanese population homozygous for the 138G variant allele. In the proceeding chapters we will show that 138G carriers have altered CD45 isoform expression, with an increased proportion of CD45R0+ T cells in the periphery. We have further shown that this polymorphism is a cause of altered isoform expression, promoting splicing towards

the low molecular weight CD45 isoforms. The high frequency of the variant allele in the Japanese population suggests that it confers some selective advantage for survival and may modulate immune responses.

### 1.3.5 A138G and disease association

We have investigated whether the 138G variant allele is associated with thyroid autoimmune disorders (Hashimoto's thyroiditis and Graves disease) and viral infections (Hepatitis B and Hepatitis C) in cohorts of Japanese patients (Boxall *et al.*, 2004). In Hashimoto's thyroiditis, humoral and cellular responses to thyroid antigens lead to the destruction of the organ and hypothyroidism. Graves disease is characterised by hyperthyroidism caused by stimulatory anti-thyrotropin receptor antibodies. Of the 126 Hashimoto's patients tested the number of A138G heterozygous individuals was found to be comparable to that in the control population. However, no G138G homozygous individuals were found despite an expected frequency of 5 G138G individuals according to the Hardy-Weinberg Law. Of the 175 patients tested who had Graves disease, the frequency of A138G heterozygote individuals was significantly reduced, and no G138G homozygous individuals were found (Table 1.2). This suggests that there may be a dominant protective effect of the 138G allele in Graves disease. This possible protective effect was also observed in the 113 hepatitis B carriers tested, with a reduced number of A138G heterozygous individuals (allele frequency of 11.9% compared to 23.7% in the control population) and only 2 G138G homozygous individuals found. There was no significant difference in the 138G allele frequency between the hepatitis C and control populations. Whilst these studies are relatively small and more data is required to fully understand the disease associations of the 138G variant allele, it does suggest that this polymorphism can have a protective effect in some diseases.

| Disease Group | Total number | A138A | A138G (allele frequency %) | G138G |
|---|---|---|---|---|
| Control | 176 | 111 | 65 (23.7%) | 9 |
| Hashimoto | 126 | 76 | 50 (19.8%) | 0* |
| Graves | 175 | 144 | 31 (9%)** | 0 |
| Hepatitis B | 113 | 88 | 23 (11.9%)*** | 2 |
| Hepatitis C | 173 | 117 | 48 (19.4%) | 8 |

**Table 1.2 Frequency of CD45 exon 6 A138G alleles in control and disease groups.** Statistically significant differences from controls are indicated by the symbols as follows. *p =0.02 (departure from the Hardy-Weinberg equation with a Chi-Square for 2df of 7.81), **p<0.001 (with a Chi-Square of 15.4 for 1df, which corresponds to a relative risk for the heterozygotes of 0.44) and ***p<0.005 (with Chi-Square of 9.3 for 1df, which corresponds to a relative risk of 0.55).

### 1.3.6 Other CD45 Polymorphisms

Jacobsen *et al.*, (2002) have described another point mutation in exon 4 of CD45, which presents a similar T-cell phenotype to that observed in individuals with the C77G polymorphism. This is a C to A transversion at position 59, which alters the amino acid sequence (histidine to glutamine), interfering with alternative splicing and resulting in expression of a structurally altered CD45 molecule. Whilst this was the first mutation to be described in the *PTPRC* gene which actually alters CD45 structure, it has so far only been described in five out of nine members of the same family. This variant was identified as part of an MS study, and found in one of 311 MS patients and in none of the 206 healthy donors.

Several other polymorphisms have recently been described in the PTPRC gene; C77T (Pro59Pro) in exon 4, G69C (Asp121His) in exon 5, and T127A (Ile187Asn) in exon 6 (Gomez-Lira *et al.*, 2003). These new polymorphisms were found in Northern Italy at frequencies of around 0-1% and nothing is currently known about their effects on CD45 alternative splicing (Gomez-Lira *et al.*, 2003). Two novel polymorphisms have lately been described in exon 4; A54G (Stanton *et al.*, 2004) and A32G (D.Pretorius, personal communication), which will be discussed further in Chapter 6.

### 1.3.7 Therapeutic applications

The essential role of CD45 in lymphocyte function and its association with various diseases makes CD45 an attractive target for immunointervention. Modulation of CD45 may affect autoimmunity, immunodeficiency, cancer and transplantation. The two main approaches to the modulation of CD45 function are the use of selective inhibitors, or specific anti-CD45 antibodies. Inhibitors of CD45 include non-selective protein tyrosine phosphatase inhibitors, such as Sulfotyrosyl peptides, Preoxtnitrite and Nitroarylhydroxymethylphosphonic acids (Beers *et al.*, 1997; Desmarais *et al.*, 1998; Takakura *et al.*, 1999). Specific CD45 inhibitors include TU752, which may be useful in the treatment of allergic disease as it inhibits IgE-mediated anaphylaxis and murine contact hypersensitivity *in vivo* (Hamaguchi *et al.*, 2001)

Monoclonal antibodies could be used to deliver targeted therapy in the form of antibody mediated cellular toxicity, cytotoxic reagents or radiation. Anti-CD45 antibodies have been shown to have an anitleukemic effect when used unconjugated or attached to radioactive iodine (Nemecek and Matthews, 2002). The anti-CD45 monoclonal antibody MG23G2 and radioactive anti-CD45 antibodies have been used to prevent allograft rejection and induce donor specific tolerance in murine models and humans (Lazarovits *et al.*, 1996; Auersvald *et al.*, 1997) MG23G2 has also been shown to have immunosuppressive ability, being used in the treatment of preclinical models of autoimmunity (Zhong and Lazarovits, 1998) Whilst its precise mechanism of action remains unclear, with suggestions including up-regulation of CTLA-4 (Fecteau *et al.*, 2001), the MG23G2 antibody is a clear candidate for further study.

### 1.4.0 Objectives of study

Although CD45 alternative splicing is tightly regulated and highly conserved in fish, birds and mammals, the exact function of the different isoforms remains obscure. Despite possible associations with a number of lectin like molecules, no specific ligand for CD45 has been identified (Perillo *et al.*, 1995; Uemura *et al.*, 1996; Symons *et al.*, 2000). In transfected cell lines it has been shown that a large extracellular domain is required for TCR signalling (Irles *et al.*, 2003). Formation of homo- and heterodimers has been proposed as a mechanism for regulating CD45 phosphatase activity (Xu and Weiss, 2002) but, the precise link between expression of different isoforms to T cell function and regulation of CD45 phosphatase activity remains unclear.

The aim of this project is to understand how the expression of alternatively spliced CD45 isoforms affects the function of leukocytes. We have utilised the existence of human CD45 polymorphisms to study the effect of altered CD45 isoform expression on T cell function and investigate the mechanisms responsible for altered immune function. Therefore our specific objectives were to:

1. Characterise CD45 isoform expression and phenotypic changes in leukocytes from individuals with variant CD45 alleles
2. Identify functional changes in T lymphocytes from individuals with variant CD45 alleles
3. Determine the molecular mechanisms responsible for altered isoform expression in 138G variant individuals
4. Characterise the CD45 isoform expression in individuals with 'novel' CD45 polymorphisms
5. Define and analyse the leukocyte phenotype and function in a transgenic mouse model which approximates abnormal CD45 splicing observed in humans

Understanding how expression of different CD45 isoforms affects immune function is important as it may lead to the development of therapeutic agents targeting CD45 and the signalling pathways that it modulates. It may also provide further insights into the function of naive and memory cells, which express different CD45 isoforms, and lead to new strategies for vaccination or immunomodulation.

# CHAPTER 2

## Materials and Methods

### 2.1.0 Materials

### 2.1.1 List of reagents and kits

| Reagent | Company |
|---|---|
| 100 bp DNA ladder | Invitrogen Life Technologies |
| 1kb DNA ladder | Invitrogen Life Technologies |
| Agarose | Invitrogen Life Technologies |
| Ammonium Acetate | Sigma |
| Ampicillin | Sigma |
| Bovine serum albumin (BSA) | Sigma |
| Bromophenol blue | Biorad |
| Chloroform | Sigma |
| Concanavalin A (ConA) | Sigma |
| Chromogenic Alkaline Phospatase substrate | BioRad |
| Cytokine Bead Array Kit (Th1/Th2) | BD Pharmingen |
| Diethyl pyrocarbonate (DEPC) | Sigma |
| Dimethyl sulphoxide | Sigma |
| Duoset ELISA development system | R&D systems |
| Dulbecco's Modified Eagle Medium (DMEM) | Gibco |
| Ultrapure dNTP | Amersham Biosciences |
| Ethylenediamiinetetraacetic acid (EDTA) | Sigma |
| Ethanol | BDH |
| Ethidium bromide | Sigma |
| Ficoll Paque PLUS | Amersham Biosciences |
| First strand cDNA synthesis Kit | Amersham Biosciences |
| Foetal calf serum | Gibco |
| Formaldehyde | BDH |
| Geneticin (G-418 Sulphate) | Gibco |

| | |
|---|---|
| HEPES (N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]) | Sigma |
| Heparin (Pump-hep with sodium) | Leo Laboratories Ltd |
| Human serum (male AB) | Sigma |
| Iscove's Modified Dulbecco's Medium (IMDM) | Gibco |
| Ionomycin | Sigma |
| Isopropanol | BDH |
| Mercaptoethanol | Gibco |
| MinElute Gel Extraction Kit | Qiagen |
| Normal mouse serum | Sigma |
| Orthopermeafix | Ortho Diagnostic Systems |
| Phosphate Buffered Saline (PBS) 10x | Gibco |
| Penicillin/streptomycin | Sigma |
| *Pfu* polymerase | Stratagene |
| Phytohaemagglutinin-P (PHA-P) | Sigma |
| Phenol (Buffer Saturated) | Invitrogen |
| Plasmid maxi kit | Qiagen |
| Phorbol 12-Myristate 13-Acetate (PMA) | Sigma |
| Proteinase K | Sigma |
| QIAprep Spin miniprep kit | Qiagen |
| RiboGreen® RNA Quantification Kit | Molecular Probes |
| RNaseOUT™ | Invitrogen Life Technologies |
| RPMI-1640 (with glutamax and HEPES) | Invitrogen Life Technologies |
| Sodium Acetate | BDH |
| Sodium Citrate | BDH |
| *Taq* polymerase (& buffer, $MgCl_2$) | Promega |
| $^3$H-thymidine | Amersham |
| 10 x TAE Buffer | Invitrogen Life Technologies |
| TRI-REAGENT™ | Sigma |
| Trypsin | IAH, Compton. |
| Trypan blue | Sigma |
| Tween20 | BDH |
| Versene (0.02% EDTA in PBS) | IAH, Compton. |
| Visigel Separation Matrix | Stratagene |
| Xylene cyanol FF | Biorad |

### 2.1.2 Monoclonal Antibodies

**Table 2.1 Monoclonal antibodies to human cell surface markers**

| Antibody | Conjugate | Species | Clone | Isotype | Supplier |
|---|---|---|---|---|---|
| CD3 | APC | Mouse | HIT3a | IgG2ak | Pharmingen |
| CD3 | FITC | Mouse | HIT3a | IgG2ak | Pharmingen |
| CD4 | Biotin | Mouse | RPA-T4 | IgG1k | Pharmingen |
| CD4 | APC | Mouse | S3.5 | IgG2ak | Pharmingen |
| CD8 | Biotin | Mouse | 3B5 | IgG2a | Caltag |
| CD8 | APC | Mouse | RPA-T8 | IgG1k | Pharmingen |
| CD11a | FITC | Mouse | G43-25B | IgG2a | Pharmingen |
| CD14 | APC | Mouse | M5E2 | IgG2ak | Pharmingen |
| CD16 | Cy-Chrome | Mouse | 3G8 | IgG1k | Pharmingen |
| CD19 | APC | Mouse | HIB19 | IgG1k | Pharmingen |
| CD25 | FITC | Mouse | ACT-1 | IgG1k | DAKO |
| CD27 | FITC | Mouse | LT27 | IgG2a | Serotec |
| CD28 | FITC | Mouse | CD28.2 | IgG1k | Pharmingen |
| CD44 | FITC | Mouse | G44-26 | IgG2bk | Pharmingen |
| CD45RA | FITC | Mouse | HI100 | IgG2bk | Pharmingen |
| CD45RA | R-PE | Mouse | 4KB5 | IgG1 | DAKO |
| CD45RA | APC | Mouse | HI30 | IgG1 | Caltag |
| CD45RA | APC | Mouse | HI100 | IgG2bk | Pharmingen |
| CD45RB | FITC | Mouse | PD7/26 | IgG1k | DAKO |
| CD45RB | R-PE | Mouse | MT4 (6B6) | IgG1k | Pharmingen |
| CD45RC | Purified | Rat | YTH80.103 | IgG2b | Biosource Int. |
| CD45RO | R-PE | Mouse | UCHL1 | IgG2ak | Pharmingen |
| CD45RO | FITC | Mouse | UCHL1 | IgG2ak | Pharmingen |
| CD45RO | APC | Mouse | UCHL1 | IgG2ak | Pharmingen |
| Pan45 | R-PE | Mouse | HI30 | IgG1k | Pharmingen |
| Pan45 | FITC | Mouse | HI30 | IgG1k | Pharmingen |
| CD56 | APC | Mouse | B159 | IgG1k | Pharmingen |
| CD56 | PE | Mouse | B159 | IgG1k | Pharmingen |
| CD62L | FITC | Mouse | Dreg56 | IgG1k | Pharmingen |
| CD62L | FITC | Mouse | LAM1-116 | IgG2ak | Ancell |
| CD69 | FITC | Mouse | FN50 | IgG1k | Pharmingen |
| CD95 | FITC | Mouse | DX2 | IgG1k | Pharmingen |
| HLA-DR | RPE | Mouse | HK14 | IgG2a | Sigma |
| CCR7 | Purified | Mouse | 2H4 | IgM | Pharmingen |

**Table 2.2 Monoclonal antibodies to human intracellular molecules**

| Antibody | Conjugate | Species | Clone | Isotype | Supplier |
|---|---|---|---|---|---|
| Perforin | FITC | Mouse | δG9 | IgG2b | Pharmingen |
| Granzyme A | FITC | Mouse | CB9 | IgG1 | Pharmingen |
| IFNγ | FITC | Mouse | 25723.11 | IgG2b | Pharmingen |
| IL4 | PE | Mouse | 3010.211 | IgG1 | Pharmingen |
| IL10 | PE | Rat | JES3-9D7 | IgG1 | Pharmingen |

**Table 2.3 Monoclonal antibodies to murine cell surface markers**

| Antibody | Conjugate | Species | Clone | Isotype | Supplier |
|---|---|---|---|---|---|
| CD3 | FITC | Armenian Hampster | 145-2C11 | IgG1k | Pharmingen |
| CD4 | FITC | Rat (Lewis) | GK1.5 | IgG2bk | Pharmingen |
| CD8 | PE | Rat (LOU/Ws1/M) | 53-6.7 | IgG2ak | Pharmingen |
| CD8 | PcP | Rat (LOU/Ws1/M) | 53-6.7 | IgG2ak | Pharmingen |
| CD19 | FITC | Rat (Lewis) | 1D3 | IgG2ak | Pharmingen |
| CD44 | PE | Rat | IM7 | IgG2bk | Pharmingen |
| CD45 | Cyc | Rat (LOU/Ws1/M) | 30-F11 | IgG2bk | Pharmingen |
| CD62 | PE | Rat | MEL-14 | IgG2a | Caltag |
| IgM | PE | Rat | LO-MM | IgG2a | Pharmingen |
| IgD | FITC | Mouse | 217-170 | IgG1k | Pharmingen |

**Table 2.4 Secondary reagents**

| Antibody | Conjugate | Species | Supplier |
|---|---|---|---|
| Streptavidin | PCP | | Pharmingen |
| Streptavidin | APC | | Pharmingen |
| Streptavidin | PE | | Pharmingen |
| Streptavidin | FITC | | Pharmingen |
| Anti-mouse IgM | FITC | Goat | Pharmingen |
| Anti-mouse IgM | PE | Goat | Pharmingen |
| Anti-Rat IgG | FITC | Goat | Pharmingen |

**Table 2.5 Isotype controls**

| Antibody | Conjugate | Species | Clone | Isotype |
|---|---|---|---|---|
| IgG2a | PE | Mouse | G155-178 | Pharmingen |
| IgG1 | PE | Mouse | MOPC-21 | Pharmingen |
| IgG1 | FITC | Mouse | MOPC-21 | Pharmingen |
| IgG2a | APC | Mouse | G155-178 | Pharmingen |
| IgG2b | FITC | Mouse | 27-35 | Pharmingen |
| IgG2a | FITC | Mouse | G155-178 | Pharmingen |
| IgM | Purified | Mouse | G155-228 | Pharmingen |
| IgG2b | Purified | Rat | R35-38 | Pharmingen |
| IgG1K | FITC | Hamster | A19-3 | Pharmingen |
| IgG2ak | FITC | Rat | R35-95 | Pharmingen |
| IgG2ak | PE | Rat | R35-95 | Pharmingen |
| IgG2ak | PcP | Rat | R35-95 | Pharmingen |
| IgG2bk | FITC | Rat | A95-1 | Pharmingen |
| IgG2bk | PE | Rat | A95-1 | Pharmingen |
| IgG2bk | Cyc | Rat | A95-1 | Pharmingen |

### 2.1.3 Media

**RPMI 1640 10% Human serum**

RPMI 1640 medium with L-alanyl-L-glutamine and 25mM HEPES

10% human serum

**RPMI 1640 10% Foetal calf serum**

RPMI 1640 medium with L-alanyl-L-glutamine and 25mM HEPES

10% foetal calf serum

**DMEM 10% Foetal calf serum**

Dulbecco's Modified Eagle Medium

10% foetal calf serum

**Complete media**

IMDM (Iscove's Modified Dulbecco's Medium)

10% FCS (heat inactivated)

50μM 2-Mercaptoethanol

100u/ml Penicillin

100μg/ml Streptomycin sulfate

2-7mM HEPES

### 2.1.4 Buffers and solutions

**TAE (10X)**

400mM Tris acetate

10mM EDTA

**PBS (per litre)**

8g NaCl

0.2g KCl

1.44g $Na_2PO_4$

0.24g $KH_2O_4$

**FACS buffer**

PBS, 0.2% BSA

**FACS Fixative**

PBS, 0.2% BSA, 1% formaldehyde

**Lysis Buffer**

25mM EDTA

10mM NaCl

10mM Tris

0.5% SDS

**TE (pH8.0)**

10mM Tris.Cl (pH8.0)

1mM EDTA

**Orange G loading buffer**

0.5% Orange G

20% Ficoll

0.5mM EDTA

Water to appropriate volume

**Blue loading buffer**

0.25% bromophenol blue

0.25% Xylene cyanol FF

30% Glycerol

### 2.1.5 Oligonucleotides

All Primers were obtained from MWG-Biotech (Germany) and were synthesised to 0.5nmol and purified by high performance liquid chromatography.

**Table 2.6 Oligonucleotides**

| Primer | Sequence (5' >3') |
|---|---|
| Exon 4 (Mut) Forward | GACTACAGCAAAGATGCCCAGTG |
| Exon 4 (Mut) Reverse | GCATTATCCAAAGAGTCCGGGG |
| Exon 2 Forward | CGAAGCTTGCTGTTTCTTAGGGACACG |
| Exon 7 Reverse | GTGAATTCCAGAAGGGCTCAGAGTGGT |
| Exon 7 Forward | ATGCCTACCTTAATGCCTCTG |
| Exon 10 Reverse | CATATTACCACACTGAAATC |
| β-actin Forward | TCGTGCGTGACATCAAAGAG |
| β-actin Reverse | TGGACAGTGAGGCCAAGATG |
| Exon 4 Forward | GGAGGAGCATACATTTAGGGT |
| Exon 4 Reverse | GTCTTTAACGTCCTTTAATCATAGAC |
| Exon 4 (Intron) Forward | CTCCTTCTCCCATTTTCCAT |
| Exon 4 (Intron) Reverse | CCCTGATATTTAAGTTAACA |
| Exon 2 Forward | TAGGGACACGGCTGGCTTCCAG |
| Exon 2A Forward | GTGGCTTAAACTCTTGGCAT |
| Exon 8 Reverse | CATGTTGGCTTAGATGGAGTAG |
| Intron 5 Forward | GTTGGCTATCTGGCTATTGCCC |
| Intron 6 Reverse | CTGAAGACACTACTAGAGCAGC |
| Exon 6 (Mutagenesis) Forward | CATCACAGCGAACGCCTCAGGTCTGAC |
| Exon 6 (Mutagenesis) Reverse | GTCAGACCTGAGGCGTTCGCTGTGATG |
| M13 Forward | GTAAAACGACGGCCAGT |
| M13 Reverse | CAGGAAACAGCTATGAC |

### 2.1.6 Plasticware

Plasticware used in laboratory work.

| Description | Company |
|---|---|
| 30ml Universal tubes | Sterilin |
| 7ml Bijou | Sterilin |
| 50ml polypropylene tubes | Falcon |
| 15ml polypropylene tubes | Falcon |
| 5ml polypropylene tubes | Falcon |
| Cryovial | Nalgene, Nunc International |
| Microamp PCR tubes | Applied Biosystems |
| Thermo-Fast 96 well PCR plates | AB gene |
| Microamp PCR caps | Applied Biosystems |
| Eppendorf tubes (1.5ml) | Anachem |
| Eppendorf tubes (0.5ml) | Anachem |
| 96-well U bottom plates (Sterile) | Nunc international |
| 96-well flat bottom plates (Sterile) | Corning, Costar |
| MutiScreen 96-well Filtration Plate (Sterile) | Millipore UK Ltd |
| 96-well maxisorb immunoplates | Nunc, International |
| PVC microtitre 96well U bottom plates | Dynex, USA |
| 25 $cm^2$ Tissue Culture treated Flasks (vented) | TPP, Helena Biosciences |
| 75 $cm^2$ Tissue Culutre Treated Flasks (vented) | TPP, Helena Biosciences |
| Cell Culture Dish (Sterile) 60mmx15mm | Corning Inc. |
| Petri Dish (Sterile) 90mmx15mm | Sterilin Ltd. |
| Gene Pulser cuvette (4mm) | BioRad |

### 2.1.7 Glassware

All glassware was provided by the Institute of Animal Health, Compton, and was autoclaved before use.

### 2.1.8 Sample Information

All assays were performed on cryopreserved PBMC. For C77G heterozygous and C77G common variant individuals, PBMC were isolated from Buffy Coat bags obtained from the National blood donor service (Colindale, North London). PBMC from all other individuals were isolated in the respective country and were cryopreseved before shipping at -20°C.

**Table 2.7 Sample Information**

| Genotype | No.of individuals | Age range (Years) | Health Status | Origin |
|---|---|---|---|---|
| C77C | 10 | 32-48 | Healthy | North London, England. |
| C77G | 10 | 36-57 | Healthy | North London, England. |
| A138A | 9 | 27-41 | Healthy | Osaka, Japan. |
| A138G | 6 | 23-30 | Healthy | Osaka, Japan. |
| G138G | 6 | 30-58 | Healthy | Osaka, Japan. |
| A54A | 2 | | HIV1+ | Uganda |
| A54G | 1 | | HIV1+ | Uganda |
| A32A | 1 | | HIV1+ | South Africa |
| A32G | 2 | | HIV1+ | South Africa |

## 2.2.0 Methods

### Cellular techniques

### 2.2.1 Isolation of peripheral blood mononuclear cells (PBMCs)

Buffy Coat bags (50ml) were obtained from the National blood donor service (Colindale, North London). When fresh blood was required, venous blood was collected in heparinised syringes with 19G x 1 inch needles (Terumo Europe, Belgium). The buffy coat/ blood samples were diluted in an equal volume of phosphate buffered saline (PBS) and slowly layered onto Ficoll Paque in 50ml Falcon tubes. The tubes were centrifuged at 500 x g, for 30 minutes at room temperature with the brake switched off. Cells were removed from the interface and washed twice with sterile PBS or appropriate media at 400 x g (10 minutes, brake on). The cells were resuspended at the required concentration in appropriate media.

### 2.2.2 Preparation of murine cells

Spleen and lymph nodes were removed from the mice and made into a single cell suspension in PBS or Complete medium by mashing and forcing through a 40μm falcon cell strainer (Becton Dickinson).

### 2.2.3 Cell viability testing

Viable cells were counted using the Trypan blue exclusion method. A small aliquot was taken from the cell suspension and diluted in an equal volume of Trypan blue solution. The live cells, as determined by the exclusion of trypan blue, were counted on a haemocytometer (improved Neubauer chamber) viewed under a light microscope (Leica). The number of cells in the suspension was calculated using the formula below.

Number of viable cells in 25 squares x Dilution factor x $10^4$ = Number of cells / ml

### 2.2.4 Cryopreservation of cells

Cells for cryopreservation were spun down and resuspended, at approximately $20x10^6$ cells/ml, in 90% foetal calf serum (FCS) and 10% dimethyl sulphoxide (DMSO). The cell suspension was transferred into cryovials in 1ml aliquots, and placed in a polystyrene box at -80°C overnight. The cells were then transferred to liquid nitrogen for long term storage.

### 2.2.5 Cell surface staining of human PBMCs

The cells ($1-2x10^5$/well) were incubated in flexible microtitre plates with 50µl of antibody, in PBS, 0.2% BSA (FACS buffer) for 20 minutes at 4°C. Antibodies were all used at pre-determined optimum concentrations. The cells were washed three times in FACS buffer at 400 x g and resuspended in an appropriate volume. If samples were to be left overnight, they were resuspended in 100µl FACS fixative buffer (PBS, 0.2% BSA, 1% formaldehyde) and stored at 4°C in the dark.

### 2.2.6 Cell surface staining of murine cells

The spleen and lymph node cell suspensions were made as described previously (Section 2.2.2). In order to lyse erythrocytes, the spleen cells were spun down and the pellet resuspended in 8ml of 0.83% Ammonium Chloride. The cells were incubated on ice for 4 minutes, before adding 20ml of PBS and passing the cells through a 0.2µm cell strainer (Falcon). The cells were spun down and resuspended in 4ml of FACS buffer.

Non-specific binding in cells isolated from spleen and lymph nodes ($2x10^5$cells/well) was blocked with either normal mouse serum or FC Block (purified anti-mouse CD16/CD32, Pharmingen) for 30 minutes at 4°C. The cells were washed in FACS buffer before incubating with a range of antibodies and appropriate isotype controls, at predetermined concentrations in a 20µl volume. The cells were incubated for 20 minutes, on ice, in the dark before washing three times at 400 x g and resuspending in an appropriate volume of FACS buffer. If samples were to be left overnight, they were resuspended in 100µl of FACS fixative buffer and stored at 4°C in the dark.

### 2.2.7 Intracellular staining

Cells were stained for cell surface markers as described previously (section 2.2.5-6) and then resuspended in 40µl of Permeafix (Ortho Diagnostics, UK) diluted 1:2 in distilled water. Following incubation for 40 minutes at room temperature in the dark, cells were washed thoroughly in FACS buffer at 400 x g. The cells were incubated with a predetermined optimum concentration of intracellular antibody, for 20 minutes, at room temperature in the dark. The cells were washed three times at 400 x g and resuspended in an appropriate volume of FACS buffer.

### 2.2.8 Intracellular cytokine staining

One hundred microlitres of cell suspension was plated out in 96-well U-bottomed plates. To each well was added 99µl of either medium alone, or medium containing PMA and Ionomycin (to give final concentrations of 50ng and 500ng/ml respectively). The Cells were incubated at 37°C, 5% $CO_2$ for 2 hours, before adding 1µl per well of GolgiPlug (BD Pharmingen) diluted 1:10 in PBS. The cells were incubated at 37°C, 5% $CO_2$ for a further 12 hours.

The cells were washed in FACS buffer, before staining for surface markers for 20 minutes on ice with the appropriate dilution of antibody. The cells were washed in FACS buffer and resuspended in 40µl Permeafix (diluted 1 in 2 in distilled water). The cells were incubated for 40 minutes (at room temperature, in the dark) and washed twice in FACS buffer. The cells were incubated with a predetermined optimum concentration of intracellular cytokine antibody for 30 minutes at room temperature, in the dark. The cells washed three times at 400 x g and resuspended in the appropriate volume of FACS buffer.

### 2.2.9 Flow Cytometery

Either freshly stained or fixed samples (with 1% formaldehyde stored in the dark at 4°C) were run on a FACSCalibur (Becton Dickinson, UK). Ten to twenty thousand events were collected and analysed using WinMDI software. Settings and compensation were established on single stained controls.

### 2.2.10 Proliferative responses of human PBMCs

A single cell suspension was plated out into 96 well flat bottom plates at $2x10^5$cells/well. Cells were stimulated with a variety of stimuli: PHA-P was used as positive control at 10μg/ml. UCHT1-2a (anti-CD3 antibody) was titrated out, and then used optimally at 1.25μg/ml. CD28 was used for co-stimulation with UCHT1-2a at 2.5μg/ml after titrating out. Tetanus Toxoid (TT) and purified protein derivative (PPD) from *M. Tuberculosis* (Statens serum institute, Denmark) were used at 5 and 10μg/ml.

Cells were incubated at 37°C, 5% $CO_2$ and tritiated thymidine ($^3H$) incorporation assays were performed on days 2-5 inclusive, for UCHT1-2a, CD28 and PHA, and on days 4-7 inclusive for Tetanus Toxoid and PPD. Cells were pulsed with 1μCi/well of $^3H$-thymidine in RPMI and plates were harvested 16 hours later. Measurement of thymidine incorporation was carried out using a Microbeta Scintillation Counter (Wallac).

### 2.2.11 Proliferative response of murine cells

96 well round bottom plates were pre-coated with CD3 (anti-mouse CD3ε chain, clone 145-2C11, no azide/low endotoxin, Pharmingen) and CD28 mAb (anti-mouse CD28, clone 37.51, no azide/low endotoxin, Pharmingen) overnight. CD3 was coated at 0, 0.1, 0.25, 0.5, 1, 5 and 10μg/ml in the presence or absence of CD28 at 2.5μg/ml. The plates were washed three times in PBS and once in medium. The cells were resuspended at $1x10^6$ cells/ml in complete medium and 200μl ($2x10^5$ cells) was added to each well. A thymidine ($^3H$) incorporation assay was performed as previously described.

### 2.2.12 Elispots

96 well filtration plates were pre-coated in anti-IFN-γ mAb (clone D1-D1K, Mabtech), anti-IL10 mAb (clone 9D7, MabTech) or anti-IL4 mAb (clone 82.4, MabTech) at 10mg/ml in sodium bicarbonate buffer, overnight. Plates are washed 4 times with sterile PBS, before adding 50μl of blocking solution (RPMI, 10% Human Serum) and incubating at 37°C, 5% $CO_2$ for 1 hour.

Cells were prepared by washing 3 times in RPMI, 15% FCS and resuspending in RPMI, 10% Human serum. 100μl of cell suspension (1-2x$10^5$cells) was added to the plates (without removing the blocking solution). PHA or UCHT1-2a was then added to a final concentration of 10 or 5μg/ml respectively. After incubating for 1 hour at 37°C, 5% $CO_2$, 30μl of filtered FCS was added to each well. The plates were then incubated at 37°C, 5% $CO_2$, for 48 hours.

The contents were removed and the plates washed four times in PBS, 0.05% Tween 20 solution. Biotin-conjugated anti-IFN-γ mAb (clone 7-B6-1-biotin, MabTech, at 1μg/ml), Biotin-conjugated anti-IL10 mAb (clone 12G8, MabTech, at 2μg/ml) or Biotin-conjugated anti-IL4 mAb (clone IL4-II (12.1), MabTech, at 2μg/ml) was added to each well as appropriate and incubated for 2 hours in a damp box at 37°C, 5% $CO_2$. The secondary antibody was removed and the plates were washed four times in PBS, 0.05% Tween 20 solution.

To each well was added 100μl of anti-biotin-ALP (Vector Laboratories) diluted 1:1000 in filtered PSB, 0.05% Tween 20, before incubating for 1-2 hours at room temperature. Plates were washed four times with PBS, 0.1% Tween 20, including the back of the filters to reduce background. 100μl of chromogenic alkaline phosphatase substrate (BioRad) was added to each well and incubated at room temperature for 30 minutes. The plates were then washed three times in water and dried before counting on an Elispot plate reader (AID).

### 2.2.13 IFNγ ELISA

The Duoset human IFNγ ELISA development system (R&D systems) was used according to the manufacturers protocol.

Briefly, 96-well plates (Maxi-sorp immunoplate, Nunc) were pre-coated with 100µl of mouse anti-human IFNγ capture antibody (diluted 4µg/ml in PBS). The plate was covered and incubated overnight at room temperature. The capture antibody was removed and the plates washed 3 times in 400µl of wash buffer (0.05% Tween 20 in PBS, pH7.2-7.4). To each well was added 300µl of Block buffer (1% BSA, 5% Sucrose in PBS with 0.05% $NaN_3$), and the plate incubated for 1 hour at room temperature before repeating the wash step as previously.

Sample and standards were diluted as appropriate in reagent diluent (0.1% BSA, 0.05% Tween 20 in Tris-buffered Saline) and 100 µl added to each well before incubating at room temperature for 2 hours. The samples were removed and the wash step repeated. 100µl of biotinylated goat anti-human IFNγ detection antibody, diluted to 100ng/ml in reagent diluent, was added to each well and incubated at room temperature for 2 hours.

The detection antibody was removed and the plate washed 3 times as previously. 100µl of Streptavidin-HRP (diluted 1/200) was added to each well and the plates incubated in the dark for 20 minutes at room temperature. The plates were washed as previously and 100µl of substrate solution (1:1 mix of $H_20_2$ and Tetramethylbenzidine) was added to each well. The plates were incubated at room temperature for 20 minutes in the dark before adding 50 µl of stop solution (2N $H_2SO_4$) to each well. The plate was tapped gently to ensure thorough mixing before immediately determining the optical density at 450nm.

### 2.2.14 Cytokine Bead Arrays

BD Biosciences Cytometric Bead Array (CBA) kits (Human Th1/Th2 and Mouse Th1/Th2 Cytokine CBA kits) were used following the manufacturer's protocol.

The Th1/Th2 cytokine standards were reconstituted (15 minutes at room temperature in 0.2ml of assay dilutent), and serial dilutions made (undiluted-1:256). For each test sample, 10µl of each cytokine capture bead suspension was mixed and 50µl of the mixed beads were transferred to each assay tube. To each assay tube was added 50µl of PE detection reagent and 50µl of diluted standard or test sample. The samples were incubated for 3 hours (human) or 2 hours (mouse) at room temperature, in the dark.

The samples were washed with 1ml of wash buffer (centrifuged at 200 x g for 5 minutes) and resuspended in 300µl of wash buffer. The standards and test samples were then run and analysed on a FACSCalibur (Becton Dickinson, UK), using CellQuest and BD CBA software, in accordance with the manufacturer's instructions.

## Molecular Techniques

### 2.2.15 DNA Isolation

Human PBMCs were isolated from suspension by centrifugation. The cell pellet (~2-5x$10^6$ cells) was re-suspended in lysis solution (500µl Lysis buffer, 10µl Proteinase K (5mg/ml) and 1µl RNase (40u/µl)). For DNA extraction from whole blood and blood clot samples, the samples were diluted with an equal volume of lysis solution. The samples were incubated at 56°C overnight. Samples not used immediately were stored at -20°C.

To each sample was added an equal volume of buffer saturated phenol. The samples were mixed and then centrifuged (12,000 x g) for one minute. The top layer was removed to a clean microcentrifuge tube and re-extracted with phenol. The top layer was removed to a clean tube and 0.2 volumes of 10M Ammonium Acetate and 2

volumes of absolute ethanol were added. The samples were centrifuged for 30 minutes (12,000 x g), before washing the DNA pellet in 70 % ethanol (7,500 x g, 5 minutes). The DNA pellet was air-dried (5 minutes) before re-suspending in 15µl of TE buffer. If not used immediately, the DNA samples were stored at -20°C.

**DNA isolation from samples in TRI-REAGENT™**

Samples were prepared as described in the RNA isolation procedure (Section 2.2.16). After removal of the aqueous phase for RNA isolation, DNA was precipitated from the interphase and organic phase by adding 0.3ml of ethanol per 1ml TRI-REAGENT™ used in sample preparation. The samples were mixed by inversion and allowed to stand at room temperature for 2-3 minutes before centrifuging (2,000 x g, 5 minutes, 4°C).

The supernatant was removed and the DNA pellet washed twice (30 minutes per wash, with occasional mixing) in 1ml 0.1M Sodium Citrate, 10% ethanol solution. After centrifugation (2,000 x g, 5 minutes, 4°C), the DNA pellet was re-suspended in 75% ethanol and stored at room temperature for 20 minutes. After centrifugation (2,000 x g, 5 minutes, 4°C), the DNA pellet was air-dried and re-suspended in an appropriate volume of TE buffer. If not used immediately, the DNA samples were stored at -20°C.

### 2.2.16 RNA Isolation

Cells were isolated from suspension by centrifugation, and washed once in PBS. The cell pellet was lysed with TRI-REAGENT™ by repeated pipetting. Lysed samples can be stored at -70°C for up to a month. One millilitre of TRI-REAGENT™ is sufficient to lyse 5-10x$10^6$ cells.

The lysed samples were centrifuged (12,000 x g, 10 minutes, 4°C) to remove the insoluble material, and transferred to a clean tube. The samples were allowed to stand at room temperature for 5 minutes before adding 0.2ml of chloroform per 1ml of TRI-REAGENT™. The samples were mixed vigorously by shaking for 15 seconds and allowed to stand for 10 minutes at room temperature before centrifuging (12,000 x g,

4°C, 15 minutes). Centrifugation separates the mixture into 3 phases, a colourless upper phase (containing RNA), an interphase (containing DNA) and a red organic phase (containing protein).

The upper aqueous phase was transferred to a clean tube and mixed with 1/10 volume of isopropanol. The samples were stored at room temperature for 5 minutes then centrifuged (12,000 x g, 10 minutes, 4°C). The supernatant was transferred to a clean tube, and the RNA was precipitated by adding 0.5ml of isopropanol per 1ml TRI-REAGENT™ used in sample preparation. The samples were stored at room temperature for 5-10 minutes, before centrifuging (12,000 x g, 10 minutes, 4°C).

The supernatant was removed and the RNA pellet was washed with 1ml of 75% ethanol in DEPC-treated water (0.1% Diethyl Pyrocarbonate) per 1ml TRI-REAGENT™ used in sample preparation. The samples were mixed before being centrifuged (7,500 x g, 5 minutes, 4°C). The RNA pellet was air-dried (5-10 minutes) before re-suspending in an appropriate volume of DEPC-treated water. RNA not immediately used was stored at -80°C.

### 2.2.17 cDNA Synthesis

Using the First strand cDNA synthesis kit (Amersham Biosciences) as per the manufacturers instructions.

One to five micrograms of total RNA was placed into a microcentrifuge tube and DEPC-treated water added to bring the volume to 8µl. The RNA solution was heated to 65°C for 10 minutes, before cooling on ice. The Bulk-First Strand cDNA reaction mix (containing: murine reverse transcriptase, RNAguard™ (porcine), RNase/DNase-free BSA, dATP, dCTP, dGTP and dTTP in aqueous buffer) was mixed by gentle pipetting to obtain a uniform suspension. Into a sterile microcentrifuge tube was placed 5µl of the reaction mix, 1µl of DTT solution (200mM), 1µl of pd(N)6 primer (0.2µg/ul random hexadeoxynucleotides) and the heat denatured RNA. The solution was mixed by pipetting before incubating at 37°C for 1 hour.

### 2.2.18 Estimation of nucleic acid concentration

**Quantitating RNA using RiboGreen®**

RNA was quantified using the RiboGreen RNA quantification Kit (Molecular probes) according to the manufacturers protocol.

The appropriate quantity of RiboGreen dye was diluted in 1xTE buffer. The Ribosomal RNA standard was made up in duplicate, in a 200µl volume (final concentration of 1, 0.5, 0.1, 0.02 and 0 µg/ml) and plated into a 96-well maxi-sorb immunoplate. Samples were plated in duplicate. To each well was added, 100µl of TE buffer, 0.5µl of RNA, and 100µl of pre-diluted ribogreen dye. The plate was read under blue fluorescence, using the Storm 860 imager (Molecular dynamics) and the RNA quantified using Quantity-One software (Bio-Rad).

**Quantification of DNA by Spectrophotometry**

DNA concentration was determined by measuring optical density (OD) at the 260nm and 280nm wavelengths. Background absorbance was set 320nm. The DNA was measured neat, or diluted as necessary in TE buffer, in a capillary tube. The DNA concentration (µg/ml) was calculated by multiplying the absorbance reading by 50 and taking into account any dilution factor. The purity of the DNA was determined using the 260/280 OD ratio, which was routinely greater than 1.8, indicating low protein contamination and sufficient purity.

### 2.2.19 Polymerase Chain Reaction

cDNA was amplified with *Taq* polymerase (in storage Buffer A; 50mM Tris-HCl pH8, 100mM NaCl, 0.1mM EDTA, 1mM DTT, 50% glycerol, 1% TritonX-100; Promega, UK). 500ng – 1µg of DNA was amplified with 1µl (10µM) specific primers. For each reaction was used 5µl 10x *taq* buffer (10mM Tris-HCL, 50mM KCL, 0.1%Triton® X-

100), 3µl 25mM $MgCl_2$, 1µl 10mM dNTP's (2.5mM each), 0.5µl T*aq* polymerase and $dH_2O$ to a final volume of 50µl.

When necessary for sequencing, PCR reactions were carried out using Pfu Turbo® DNA polymerase (in 50mM Tris-HCL (pH 8.2), 1mM DTT, 0.1mM EDTA, 0.1% Tween20 and 50% (v/v) glycerol; Stratagene). 500ng – 1µg of DNA was amplified with 1µl (10µM) specific primers. For each reaction was used 5µl 10x Reaction buffer (200mM Tris-HCL (pH8.8), 100mM KCL, 100mM $(NH_4)_2SO_4$, 20mM $MgSO_4$, 1% Triton® X-100, 1mg/ml nuclease-free BSA), 1µl 10mM dNTP's, 0.5µl Pfu Turbo® polymerase and $dH_2O$ to a final volume of 50µl.

The PCR was performed in thin-walled PCR tubes using the Perkin Elmer thermal cycler. The standard PCR conditions were as follows:

| | |
|---|---|
| 1 Cycle: | Denaturing 92°C for 4 minutes |
| 30 Cycles: | Denaturing 92°C for 1 minute |
| | Annealing 55°C for 1 minute |
| | Extension 72°C for 4 minutes |
| 1 Cycle: | Extension 72°C for 16 minutes |
| | Hold at 4°C |

The PCR conditions were altered slightly for the minigene experiments (see chapter 5). The new PCR conditions were as follows:

| | |
|---|---|
| 1 Cycle: | Denaturing 94°C for 3 minutes |
| 30 Cycles: | Denaturing 94°C for 1 minute |
| | Annealing 55°C for 1 minute |
| | Extension 72°C for 1 minute |
| 1 Cycle: | Extension 72°C for 10 minutes |
| | Hold at 4°C |

### 2.2.20 Gel Electrophoresis

#### Agarose gel electrophoresis

DNA fragment size was determined by agarose gel electrophoresis. Gels were prepared with 0.5-3% agarose (according to size) in 1xTAE buffer and ethidium bromide (0.5μg/ml) added. The samples were mixed with loading buffer at a ratio of 1:6 and loaded against 1Kb or 100bp ladders as appropriate. Electrophoresis was carried out at 50-80v for the appropriate length of time, and the DNA bands were then visualised under UV light.

#### VisiGel™ Separation Matrix

The VisiGel™ separation matrix was used to determine the fragment size of PCR products 100-1200bp in length. Gels were prepared with 3ml of VisiGel™ separation matrix concentrate per 50ml of 1xTAE buffer, and ethidium bromide (0.5μg/ml) added. The samples were mixed with loading buffer at a ratio of 1:6 and loaded against a 100bp ladder. Electrophoresis was carried out at 50-80v for the appropriate length of time, and the DNA bands were then visualised under UV light.

### 2.2.21 Purification of fragments from Agarose gels

DNA bands were extracted from 2-3% Agarose gels for subsequent cloning and / or sequencing. The DNA was visualised under UV light and excised from the gel with a clean scalpel. The DNA was extracted from the gel fragment using the Qiagen MinElute Gel Extraction Kit, following the manufacturer's protocol.

In brief, the gel fragments were dissolved in 3x the gel volume of buffer QG at 50°C for 10 minutes (with regular vortexing). Isopropanol (1x gel volume) was added and the sample mixed by inversion. The samples was applied to the MinElute column and centrifuged (10,000 x g) for 1 minute. The flow-through was discarded and 500μl of buffer GC added, before centrifuging (10,000 x g) for 1 minute. The flow-through was discarded and the column washed with 750μl of buffer PE (centrifuging for 1 minute,

10,000 x g). The flow-thorough was discarded and the column centrifuged for an additional minute (10,000 x g). The column was placed into a clean 1.5ml microcentrifuge tube and the DNA eluted by adding 10µl of Buffer EB to the centre of the column and allowed to stand for 1 minute before centrifuging (1 minute, 10,000 x g).

### 2.2.22 Preparation of Plasmid DNA

**Small scale preparation (miniprep)**

Small scale preparation of plasmid DNA was carried out using QIA Spin Miniprep kit (Qiagen, UK) following the manufacturers recommended protocol.

Bacterial cultures (3ml), picked from a single colony, were incubated overnight at 37°C. One millilitre of the bacterial culture was transferred to a sterile microcentrifuge tube. The cells were collected by centrifugation at 14,000 x g for 5 minutes before re-suspending in 250µl of buffer P1 (50mM Tris-Cl (pH8.0), 10mM EDTA, 100µg/ml RNaseA). Two hundred and fifty microlitres of lysis buffer P2 (200mM NaOH, 1% SDS) was added and the samples mixed by inversion before adding, 350µl neutralisation buffer N3. The mixed samples were centrifuged at 14,000 x g, for 10 minutes and the supernatant transferred onto a QIAprep spin column (in a 2ml collection tube). This was centrifuged at 14,000 x g for 30-60 seconds and the flow-through discarded. The column was washed in 0.5ml Buffer PB and 0.75ml Buffer PE, centrifuging as previously after each wash, with an additional spin following the buffer PE wash. DNA was eluted into a sterile microcentrifuge tube in 50µl Buffer EB (10mM Tris-Cl (pH8.5)).

**Large scale preparation (maxiprep)**

Large scale preparation of plasmid DNA was carried out using QIAfilter Plasmid Maxi Kit (Qiagen, UK) following the manufacturers recommended protocol.

Bacterial culture (3ml) prepared from a single colony was further grown in 400ml LB-medium with 100μg/ml ampicillin overnight at 37°C in a horizontal shaker at 225rpm. The cells were collected by centrifugation at 6,000 x g for 15 minutes (at 4°C). The pellet was resuspended in 10ml of buffer P1 (50mM Tris-Cl (pH8.0), 10mM EDTA, 100μg/ml RNaseA) before adding lysis buffer P2 (10ml, 200mM NaOH, 1% SDS). Samples were mixed and incubated for 5 minutes at room temperature before adding 10ml chilled neutralisation buffer P3 (3.0M potassium acetate, pH5.5), and inverting to mix. The lysate was transferred into the barrel of the QIAfilter maxi cartridge and incubated for 10 minutes at room temperature. The lysate was filtered through the maxi cartridge before applying to a pre-equilibrated (with 10ml equilibration buffer QBT (750mM NaCl, 50mM MOPS, pH7.0, 15% isopropanol; 0.15% triton)) QIAGEN-tip 500 column and allowing it to enter the resin by gravity flow.

The column was washed twice with 30ml wash buffer QC (1.0M NaCl, 50mM MOPS, (pH7.0) 15% isopropanol) and the DNA was eluted into a sterile tube in 5ml elution buffer QF (1.25M NaCl, 50mM Tris-Cl (pH8.5), 15% isopropanol). To precipitate the DNA, 0.7 volumes of isopropanol was added and the samples centrifuged at 15,000 x g for 30 minutes, before washing in 70% ethanol, air drying the pellet and re-suspending it in an appropriate volume of TE buffer.

### 2.2.23 Restriction Endonuclease Digests

Specific restriction enzymes were used to digest double stranded DNA. Enzymes were used at a concentration to give 1 to 5 units per μg of DNA. The appropriate buffers (supplied with the enzymes) were used at 1:10 dilution according to manufacturer's instructions. Reactions were incubated at 37°C for 1 to 2 hours unless otherwise recommend.

**Table 2.8 Restriction Enzymes**

| Enzyme | Sequence recognised | Buffers | Manufacturer |
|---|---|---|---|
| *EcoR1* | 5'-G/AATTC-3' | 10x $OPA^+$ | Amersham Biotech |
| *Dpn1* | 5'-GmA/TC-3' | 10x React 4 | Invitrogen |
| | | 10x T buffer | |
| *Msp1* | 5'-C/CGG-3' | 0.1% BSA | Amersham Biotech |
| | | 10x K buffer | |
| *Pvu1* | 5'-CGAT/CG-3' | 0.1% BSA | Amersham Biotech |

### 2.2.24 Site directed mutagenesis

Maxiprep DNA of the plasmid containing exons 2, 6 and 8, was linearised using the restriction enzyme Pvu 1, and then purified by adding 0.1volumes of 3M Sodium Acetate (pH5.2) and 2.5 volumes of 100% Ethanol. The samples were centrifuged at 12,000 x g for 15 minutes at 4°C, before washing in 70% ethanol, air-drying the DNA pellet and resuspending in 20µl of TE buffer.

The mutagenesis PCR reactions were carried out using Pfu Turbo® DNA polymerase (Stratagene). 500ng of DNA was amplified with 1µl (125ng) of Exon 6 mutagenesis forward and reverse primers. For each reaction was used 5µl of 10x Reaction buffer, 1µl of dNTP mix (2.5mM each), 1µl of Pfu Turbo® polymerase (2.5u/µl) were used and $dH_2O$ was added to a final volume of 50µl.

The PCR was performed in thin-walled PCR tubes using the Perkin Elmer thermal cycler. The mutagenesis PCR conditions were as follows:

| | |
|---|---|
| 1 Cycle: | Denaturing 95°C for 30 Seconds |
| 12 Cycles: | Denaturing 95°C for 30 Seconds |
| | Annealing 55°C for 1 minute |
| | Extension 68°C for 12 minutes (2min/kb plasmid length) |

The mutagenesis PCR products were then digested with Dpn1, and a small sample run out on an agarose gel to check the reaction, before transforming into XL1-blue supercompetent cells.

### 2.2.25 Transformation of *E.Coli* Strains

**XL1-Blue MR**

Plasmid was transformed in XL1-Blue MR supercompetent cells (Stratagene) using the manufacturers standard protocol.

The competent cells were thawed on ice, and mixed gently before adding 100µl of cell suspension into each pre-chilled 15ml Falcon tube. To each tube was added 1.7µl of β-mercaptoethanol (to 25mM final concentration), before incubating them on ice for 10 minutes, mixing the contents gently every two minutes. Plasmid DNA (0.1-50ng) was added and the cells incubated on ice for 30 minutes. The cells were heat pulsed at 42°C for 45 seconds, before incubating on ice for 2 minutes. To the cells was added 0.9ml of preheated (42°C) SOC medium, before incubating at 37°C, 225rpm for 1 hour. Appropriate volumes of the transformation mixture were plated onto LB plates containing 100µg/ml ampicillin and incubated for a minimum of 17 hours at 37°C. Single colonies were picked and grown up in 3ml overnight cultures ready for DNA extraction by miniprep.

**One Shot® TOP10 cells**

Cloned PCR products were transformed into One Shot® TOP10 cells (Invitrogen Life Technologies) using the manufactures standard protocol.

Two microlitres of the TOPO cloning reaction was added to a vial of One Shot® Chemically Competent *E.Coli* (TOP10) and incubated on ice for 5-30 minutes. The cells were heat shocked for 30 seconds at 42°C without shaking, and immediately transferred onto ice. Two-hundred and fifty microlitres of room temperature SOC medium was

added and the cells incubated at 37°C, 200rpm, for 1 hour. Appropriate volumes (10-50µl) of each transformation reaction were plated onto pre-warmed selective plates (LB plates containing 100µg/ml Ampicillin and pre-coated with 100µl of 2% X-gal). The plates were incubated for a minimum of 17 hours at 37°C and single white or very light blue (not dark blue) colonies were picked and grown up in 3ml overnight cultures, before extracting the DNA by miniprep.

### 2.2.26 Transfection of COS-7 cells

COS-7 cells were grown in DMEM +10% FCS until 80% confluent. The cells were harvested with 0.25% Trypsin diluted 1:8 with Versene. The cells (~$5x10^6$cells/ml) were washed once in cold PBS (centrifuging at 400 x g), and once in cold PBS + 10mM HEPES. One-hundred micro-litres of cell suspension were placed into a sterile electroporation cuvette and 1µg of linearised (*PvuI* digested) plasmid was added to the cells. For stable transfections 1µg of linearised (*EcoRI* digested) pcDNA3 was added to the cells with 2µg of linearised (*PvuI* digested) plasmid. The cells were electroporated using the BioRad Gene Pulser II (280v, 25µF, 200Ω).

To the cells was added 1ml of medium (DMEM+10%FCS+1mM Hepes). The cell suspension was transferred to a 60mm sterile tissue culture dish and a further 4ml of media was added. The cells were incubated at 37°C, 5% $CO_2$. For transient transfections the cells were harvested with Trypsin solution (0.25% Trypsin diluted 1:8 with Versene) after 48 hours. For stable transfections, geneticin (G418 sulphate) was added to the cells (5-10mg/ml) after 48 hours. The cells were maintained in this selective medium for a further two weeks until stable clones had been established. The stably transfected cells were harvested and washed once in PBS before being resuspended in TRI-REAGENT™ for RNA extraction.

### 2.2.27 Cloning PCR products

Using the TOPO TA cloning® kit (Invitrogen life technologies), according to the manufacturers protocol.

The cloning reaction was set up with 0.5-4µl of fresh PCR product, 1µl of salt solution, 1µl of TOPO® Vector and sterile water to a total volume of 5µl. The reaction was incubated at room temperature (22-23°C) for 5 minutes before placing on ice. The cloning reaction was then transformed into chemically competent TOP10 cells (See transformation of *E.Coli* strains).

### 2.2.28 Sequencing

DNA samples were sent for sequencing with the appropriate custom primers. All sequencing reactions were carried out by Lark Technologies Inc, Essex, UK.

### 2.2.29 Statistical Analysis

Chi-square test, using Yates continuity correction to allow for small numbers, was used to analyse the disease association of the 138G variant allele. For comparison of the phenotypic analysis between cell subsets in CD45 variant and control individuals, the Mann-Whitney test was used. Analysis of variance (ANOVA) was carried out to determine the significance of the observed difference between the proliferative responses of PBMC from CD45 variant and control individuals. All statistical analysis was carried out using MINTAB statistical software, version 13.1, MINITAB Ltd., USA.

# CHAPTER 3

## Effect of variant CD45 expression on cell phenotype

### 3.1 Introduction

An essential aspect of immune function is the differentiation of lymphoid cells in the periphery. Alterations in the expression of various surface markers, including molecules involved in adhesion, co-stimulation and migration are critical in the homing of lymphocytes and the activation of the immune response. Changes in expression are related to the different properties of naïve, effector and memory cells (as discussed in Chapter 1) and have commonly been used to define these different populations.

The expression of different CD45 isoforms is cell type specific and dependant upon the activation and differentiation state of the cells (Akbar *et al.*, 1988; Powrie, 1990). Following activation human T cells are found to lose expression of the high molecular weight isoforms containing the A exon and gain expression of the low molecular weight CD45R0 isoform. For this reason CD45 isoform expression is often used to distinguish naïve (CD45RA+) and memory (CD45R0+) T cells. Despite this, little is known about the exact function of the different CD45 isoforms. It has been shown that CD45 knockout mice and humans lacking CD45 expression are severely immunodeficient with few peripheral T lymphocytes (Kishihara *et al.*, 1993; Byth *et al.*, 1996; Kung *et al.*, 2000; Tchilian *et al.*, 2001). Therefore it is important to establish whether variant CD45 isoform expression has an effect on the proportions of different cell types or on T cell phenotype.

Schwinzer and Wongeit (1990) first observed a group of individuals with constitutive CD45RA expression on their T cells even after mitogen stimulation. This variant CD45 expression was later associated with the exon 4 C77G polymorphism, which disrupts a strong exonic splice silencer, ESS1, that usually represses the inclusion of exon 4 (Thude *et al.*, 1995; Zilch *et al.*, 1998; Lynch and Weiss, 2001). Recently a more

prevalent polymorphism has been described in exon 6, with the A138G variant being present at an allele frequency of 23.7% in the Japanese population. One of the aims of this chapter is to determine the effects of the 138G variant allele on CD45 isoform expression. Using some of the important cell surface markers for characterising naïve and memory cells we will also investigate the altered CD45 isoform expression on other aspects of lymphocyte phenotype.

T cell activation results in changes in the expression of cell surface markers including molecules involved in activation (CD25, CD69, HLA-DR), co-stimulation (CD27, CD28) and adhesion (CD11a, CD62L, CD44) as well as cytokine receptors such as CD25 and CCR7. CD69 is one of the earliest antigens to emerge upon activation of lymphocytes, appearing within two hours of stimulation. Although not expressed on resting lymphocytes, it is rapidly induced upon activation of T cells, B cells and NK cells. There are a number of markers for activation including MHC Class II molecules, which are expressed on various cells including dendritic cells, B cells, monocytes, and macrophages. HLA-DR (MHC Class II) is expressed on activated T cells. Expression is regulated by cytokines, including Interferon gamma. A marker of activation and a cytokine receptor, CD25 is the α-chain subunit of the IL-2 receptor, which is expressed on activated T cells, B cells and mononcytes. IL-2 is an important cytokine in the activation and proliferation of T cells, thymocytes, NK cells, B cells and macrophages.

CD27 is a member of the tumour necrosis factor receptor family, and is known to be a co-stimulatory molecule on T cells. It is expressed on naïve cells, but down regulated on chronically activated cells, decreasing with cell division after activation. Work on CD27-/- mice suggest that it is important in memory responses, particularly in the responses of primed CD8+ cells to influenza virus (Hendriks *et al.,* 2000). CD28 is another co-stimulatory molecule which is constitutively expressed on most T lineage cells and plasma cells. Generally, activation of T cells leads to enhanced CD28 expression, while ligation of CD28 leads to its transient down-regulation. However, loss of CD28 is also associated with increasing differentiation, particularly among CD8 cells.

CD11a, or the Leukocyte function antigen – 1 (LFA-1), is an adhesion molecules that is expressed on lymphocytes, granulocytes, monocytes and macrophages, with higher expression levels on memory T cells. CD11a is an integrin αchain, which is expressed as a heterodimer non-covalently associated with CD18 (integrin β2). It mediates cell-cell and cell-matrix adhesion, so is important in many leukocyte functions, including proliferation and the interaction of leukocytes with tissues, such as the endothelium. Expression of the adhesion molecule CD44 is increased on memory T cells. It is the principal cell surface receptor for hyaluronate, and this interaction mediates binding of lymphocytes to high endothelial venules (HEV's). Another cellular adhesion molecule important for the recirculation of lymphocytes through lymphoid tissue is CD62L or L-Selectin. It is involved in the initiation of rolling and the adhesion of lymphocytes to specialised HEV's in lymph nodes and Peyer's patches. Whilst found on most resting lymphocytes, it is rapidly shed upon T cell activation (Chao *et al.,* 1997). However, it can then be re-expressed and increased expression levels are seen after culturing in medium (Wallace and Beverley, 1993).

The chemokine receptor CCR7 is also involved in the control of lymphocyte homing to secondary lymphoid tissues. CCR7 has been used to divide human memory T cells into two functionally distinct subsets. These are CCR7+ central memory cells, which express lymph node homing receptors and lack immediate effector function and CCR7- effector memory cells which express receptors for migration to inflamed tissues and have immediate effector function (Sallusto *et al.,* 1999). Expression of CCR7 is often correlated with that of other adhesion molecules such as CD62L and the combined loss of CCR7 and CD62L is predicted to be responsible for the loss of homing to lymphoid tissues of effector memory T cells.

## Objectives

The first objective of this chapter is to characterise the specific isoforms and level of CD45 expression, on different subsets of cells from individuals with the exon 4 C77G and exon 6 138G variant alleles. The second objective of this chapter is to investigate the phenotype of T cells from individuals with variant CD45 expression.

## 3.3 Results

### 3.3.1 The C77G Polymorphism

#### 3.3.1.1 Variant CD45 splicing

PBMC were isolated from Buffy Coat bags obtained from the National blood donor service (Colindale, North London). All of the samples were screened for the presence of the exon 4 C77G polymorphism by flow cytometry and confirmed by PCR amplification of genomic DNA and *MspI* digestion or direct sequencing. All of the assays were performed on cryopreserved PBMC.

In accordance with the previously published data (Schwinzer and Wonigeit, 1990) flow cytometric analysis of CD45RA and CD45R0 expression on T cells from C77G heterozygous individuals shows a distinct expression pattern (Figure 3.1a), with the absence of single positive CD45R0 cells. Whilst normal CD45 expression is characterised by the loss of CD45RA and the gain of CD45R0 expression upon activation, the lymphocytes from C77G heterozygous individuals are characterised by a lack of single CD45R0+ cells, even after stimulation (Figure 3.1b).

The C77G transversion introduces a new restriction site for the *MspI* endonuclease (C/CGG). Therefore the presence of the polymorphism can also be detected by the PCR amplification of genomic DNA (using Exon 4 (Mut) forward and reverse primers, see Chapter 2) and digestion of the PCR product with MSP1. Digestion with MSP1 cleaves that mutant PCR product into two fragments of 72 and 83 base pairs (bp) as can be seen in Figure 3.2. All of the individuals identified so far have been heterozygous for the C77G polymorphism, so the presence of the 77C wild-type allele is shown by the un-cleaved 155bp PCR product.

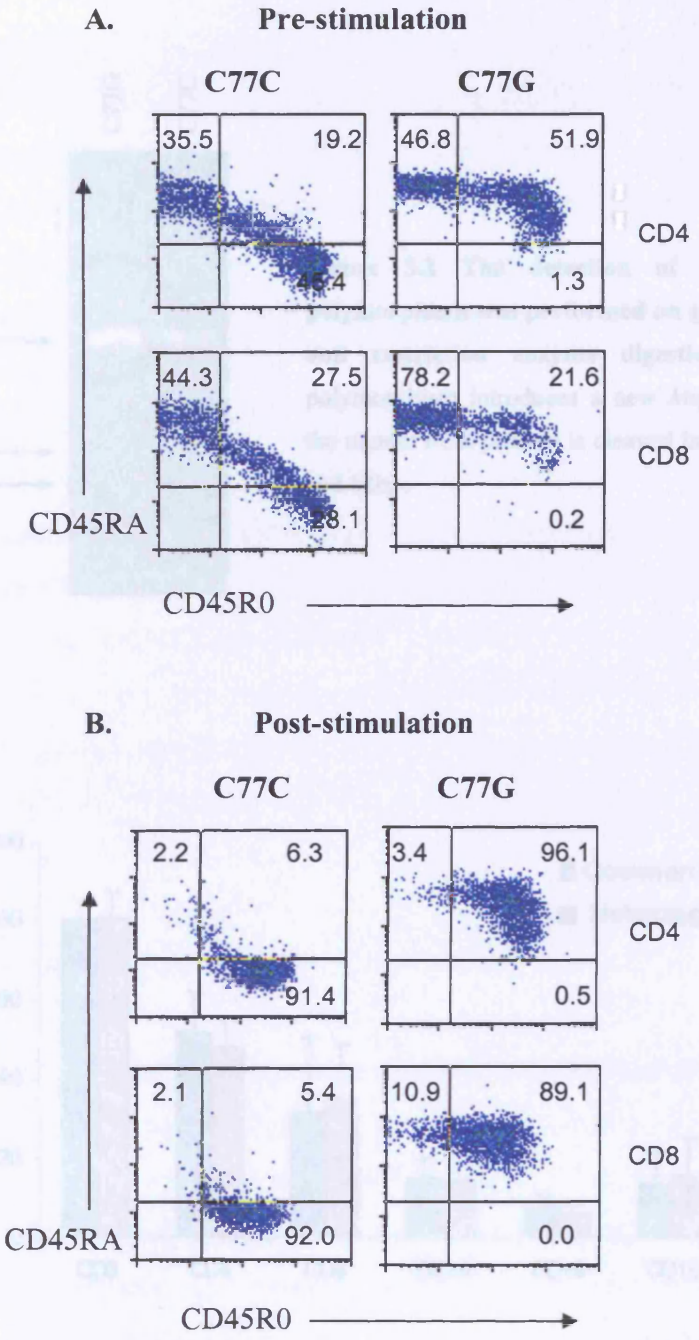



**Figure 3.1 Flow cytometric analysis of exon 4 C77G and control individuals.** PMBCs were stained with CD45R0-PE, CD45RA-FITC and either CD4 or CD8-APC antibodies pre and post-stimulation with 10μg/ml PHA for 10 days. Analysis was performed on CD4 and CD8 gated T cells. Normal expression is characterised by the loss of CD45RA and gain of CD45R0 expression upon activation. Variant CD45 expression is characterised by the absence of the single CD45R0+ population even after stimulation.

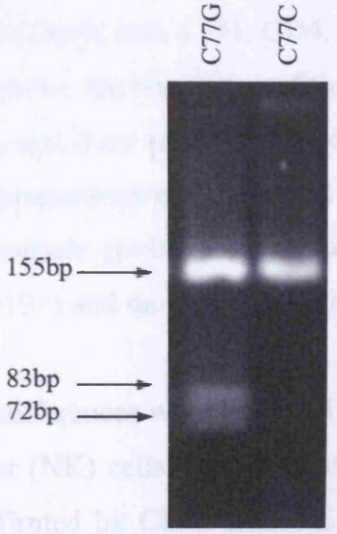



**Figure 3.2 The detection of the exon A C77G polymorphism was performed on genomic DNA by PCR and restriction enzyme digestion with *MspI*.** The polymorphism introduces a new *MspI* restriction site, and the mutant PCR product is cleaved into two fragments of 72 and 83bp.

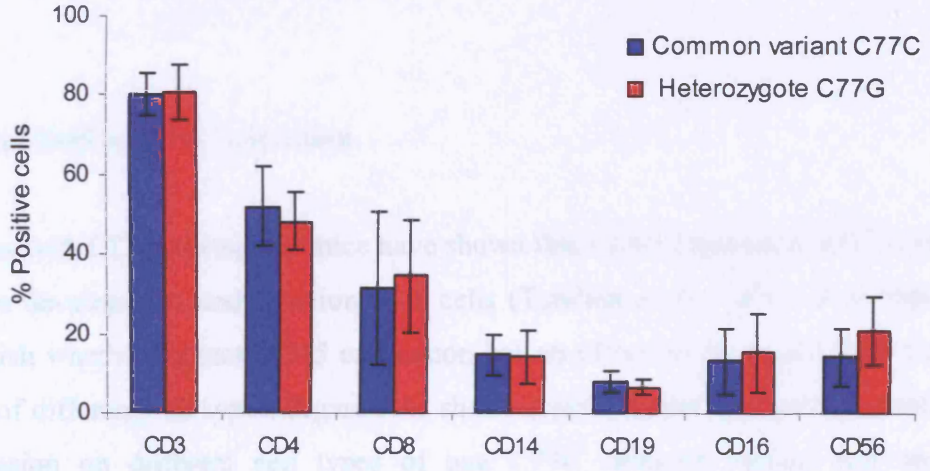



**Figure 3.3 Percentage of different populations of cells in PBMC from common variant C77C and heterozygous C77G individuals.** Mean and standard deviation of ten individuals per group.

### 3.3.1.2 Cell populations

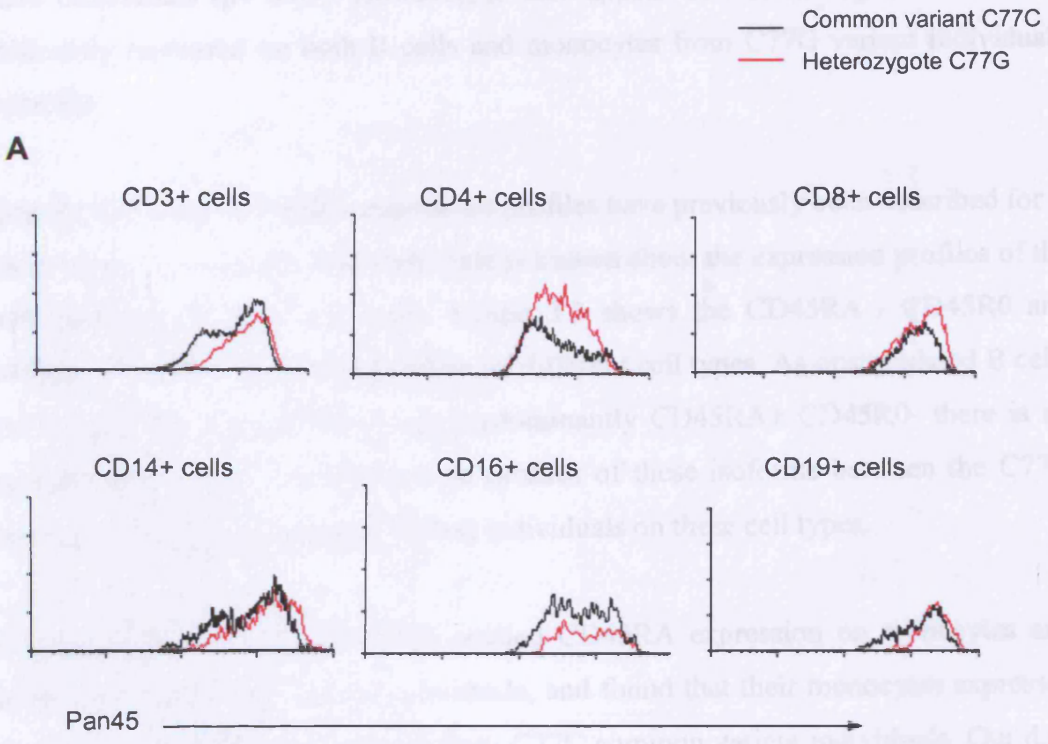
PBMC from healthy C77C common variant and C77G heterozygous individuals were stained with anti- CD3, CD4, CD8, CD14, CD16, CD19 and CD56 antibodies. Figure 3.3 shows the proportion of the different cell types (mean of ten individuals per group). Although there is some individual variation, no significant differences were observed in the proportions of either CD4+ or CD8+ T cells between the C77C and C77G variant individuals ($p>0.1$ for all markers). This is also true for the proportions of B cells (CD19+) and monocytes (CD14+).

No differences were observed in the proportions of CD16+ cells, which include natural killer (NK) cells, macrophages and mast cells. The proportions of NK cells were also confirmed by CD56 staining, and no significant differences were found in either the proportion of classical NK cells (CD56+ CD3- cells) or NK T cells (CD56+CD3+ cells) (data not shown). This data suggests that the variant CD45 expression caused by the C77G polymorphism does not have an effect on the proportions of different populations of cells.

### 3.3.1.3 CD45 isoform expression

Studies with CD45 transgenic mice have shown that CD45 expression level is important for the development and function of T cells (Tchilian *et al.,* 2004). It is important to establish whether variant CD45 expression has an effect on the total CD45 expression level of different cell types. Figure 3.4a shows representative histograms of total CD45 expression on different cell types of one C77C common variant and one C77G heterozygous individual. These cells were stained with PanCD45, a monoclonal antibody against an epitope present on all CD45 isoforms. Interestingly there does appear to be a slight shift in the total CD45 expression level, with the T cells, B cells and monocytes from C77G heterozygous individuals staining slightly brighter (Figure 3.4b).

Despite the observable trend, large individual variation means that there is no significant difference in the CD45 expression level on T and NK cells from C77G




B

| Cell Type | CD45 Expression (Mean Fluorescence Intensity) C77G | C77C |
|---|---|---|
| CD3+ | 942 +/- 360 | 620 +/- 304 |
| CD4+ | 795 +/- 293 | 539 +/- 159 |
| CD8+ | 1089 +/- 282 | 945 +/- 435 |
| CD14+ | 1738 +/- 505* | 1048 +/- 247 |
| CD16+ | 786 +/- 126 | 617 +/- 269 |
| CD19+ | 1661 +/- 413* | 1176 +/- 258 |

**Figure 3.4 Total CD45 expression on a range of different cell types.** PBMC were stained with Pan45-PE (a monoclonal antibody against an epitope present on all CD45 isoforms) and a range of markers for different cell types. (A) Histograms showing one representative individual from each group of 6 C77C common variant individuals (Black lines) and 6 C77G heterozygous individuals (Red lines). (B) Mean Fluorescence intensity of pan45 staining on each cell type. Mean of 6 individuals per group, with standard deviation. Differences between C77G homozygous and C77C common variant individuals was analysed using the Mann-Whitney test: * p= 0.030.

variant individuals ($p > 0.05$). However, it does appear that CD45 expression level is significantly increased on both B cells and monocytes from C77G variant individuals ($p = 0.030$).

Whilst the CD45R0 / CD45RA expression profiles have previously been described for T cells in variant individuals, relatively little is known about the expression profiles of the CD45 isoforms on other cell types. Figure 3.5 shows the CD45RA / CD45R0 and CD45RB / CD45RA expression profiles on different cell types. As unstimulated B cells (CD19+) and NK cells (CD56+) are predominantly CD45RA+ CD45R0- there is no observable difference in the expression profiles of these isoforms between the C77G heterozygous and C77C common variant individuals on these cell types.

Previously, Schwinzer *et al.,* (1992) studied CD45RA expression on monocytes and granulocytes from C77G variant individuals, and found that their monocytes expressed higher levels of CD45RA than those from C77C common variant individuals. Our data also show no observable difference in the CD45R0 / CD45RA expression pattern on monocytes (CD14+), which are all double positive (CD45RA+ CD45R0+). However, in accordance with the previous study it does appear that the CD45RA expression is slightly brighter on monocytes from the C77G variant individuals. Schwinzer *et al.*, also described the granulocytes from variant individuals as CD45RA+ when granulocytes from C77C common variant individuals did not express the CD45RA antigen. This may be an interesting area for further investigation, but due to sample constraints was not covered in this investigation.

The only obvious difference in the CD45R0 / CD45RA expression pattern is seen on T cells, with CD8+ T cells being particularly affected. When looking at the CD45RA / CD45RB expression pattern, it can again be seen that the cells from C77G heterozygous individuals are always CD45RA+, and correspondingly have less CD45RB low cells (Figure 3.5). This increase in the proportion of CD45RB high cells is predominantly observed in T cells, whilst other cell types such as B and NK cells which are generally found to be CD45RB high are not affected. CD45 isoform expression as determined by message level will be further discussed in Chapter 5.

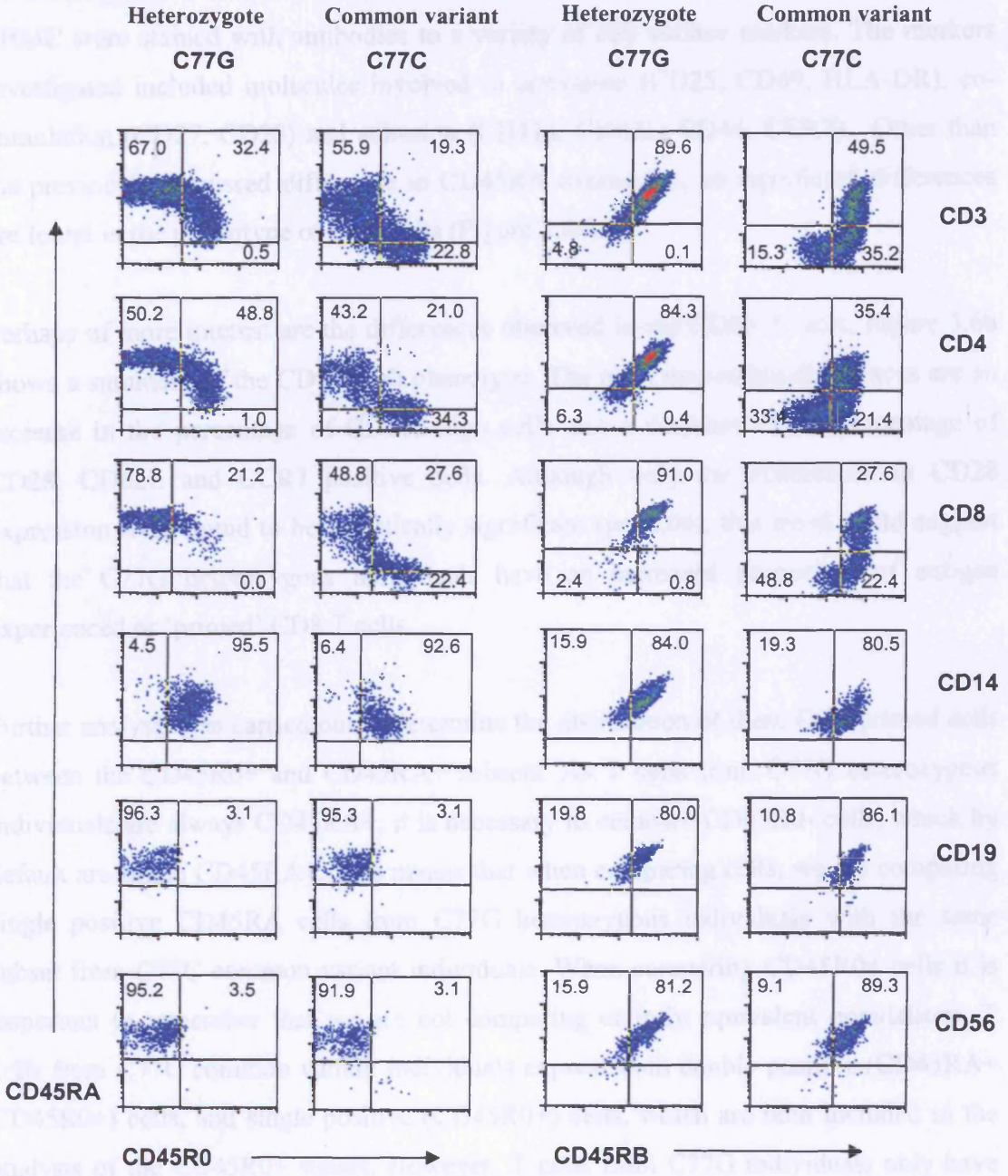



**Figure 3.5 CD45 isoform expression on different cell types.** PBMC were stained with a range of cell type specific antibodies with isoform specific CD45R0, CD45RA and CD45RB antibodies. Profiles are shown gated on a different cell types. Examples are representative of similar analysis on 6 C77C common variant individuals and 6 C77G heterozygous individuals.

### 3.3.1.4 T cell phenotype

To investigate the effects of C77G variant allele on other aspects of T cell phenotype, PBMC were stained with antibodies to a variety of cell surface markers. The markers investigated included molecules involved in activation (CD25, CD69, HLA-DR), co-stimulation (CD27, CD28) and adhesion (CD11a, CD62L, CD44, CCR7). Other than the previously discussed difference in CD45RA expression, no significant differences are found in the phenotype of CD4 cells (Figure 3.6a).

Perhaps of more interest are the differences observed in the CD8+ T cells. Figure 3.6b shows a summary of the CD8 T cell phenotype. The most interesting differences are an increase in the percentage of CD11a high cells and a decrease in the percentage of CD28, CD62L and CCR7 positive cells. Although only the differences in CD28 expression were found to be statistically significant ($p= 0.04$), this trend could suggest that the C77G heterozygous individuals have an increased proportion of antigen experienced or 'primed' CD8 T cells.

Further analysis was carried out to determine the distribution of these CD8 primed cells between the CD45R0+ and CD45RA+ subsets. As T cells from C77G heterozygous individuals are always CD45RA+, it is necessary to compare CD45R0- cells, which by default are single CD45RA+. This means that when comparing cells, we are comparing single positive CD45RA cells from C77G heterozygous individuals with the same subset from C77C common variant individuals. When comparing CD45R0+ cells it is important to remember that we are not comparing entirely equivalent populations. T cells from C77C common variant individuals express both double positive (CD45RA+ CD45R0+) cells, and single positive (CD45R0+) cells, which are both included in the analysis of the CD45R0+ subset. However, T cells from C77G individuals only have double positive (CD45RA+ CD45R0+) cells. With CD8 cells in particular this comparison is further complicated by the disparity in the proportions of the different populations (Figure 3.1a).

Figure 3.7 shows the further analysis of CD4 and CD8 cells in both the CD45R0+ and CD45R0- subsets. No real differences were observed in the CD4 cells other than a slight alteration in the proportion of CCR7+ cells. Figure 3.7b indicates that there is a slight

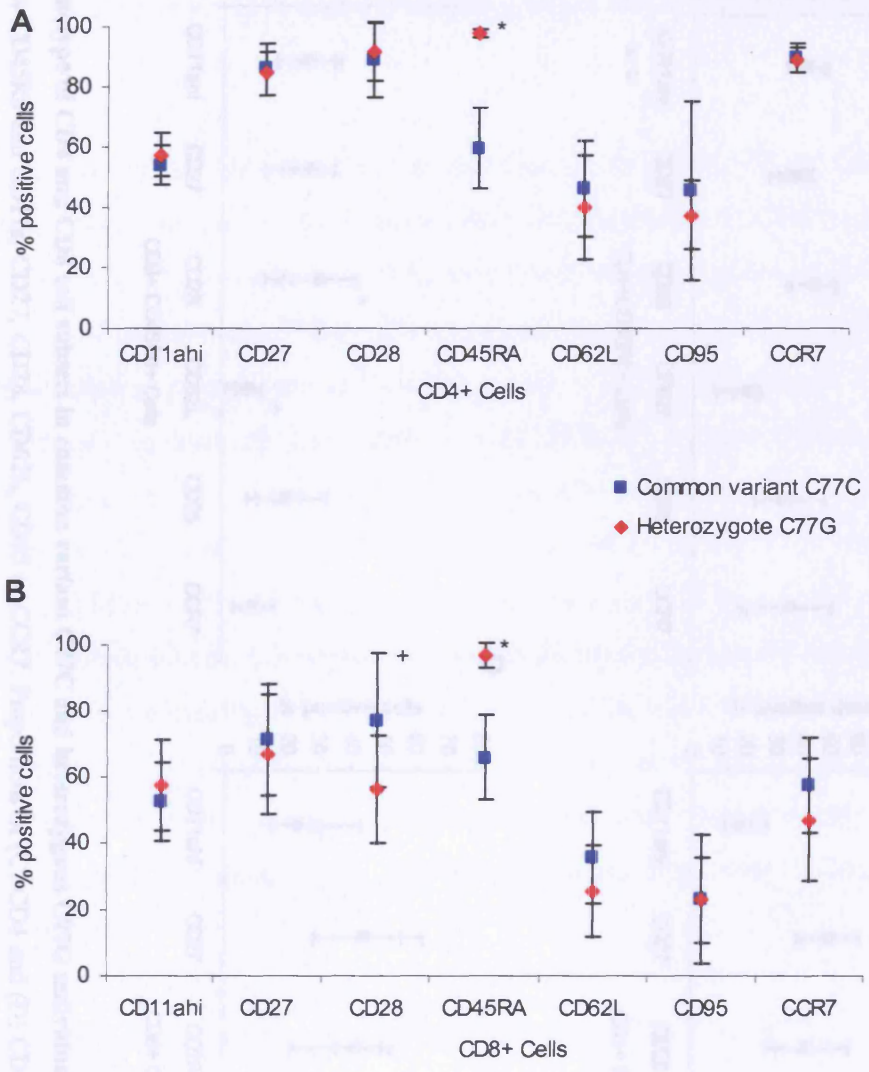



**Figure 3.6 Phenotype of CD4 and CD8 cells in common variant C77C and heterozygous C77G individuals.** PBMC were stained with CD4 or CD8 and CD11a, CD27, CD28, CD45RA, CD62L, CD95 and CCR7. Proportions of (A) CD4 and (B) CD8 T cells expressing different markers. Data shows means and standard deviation of 10 C77C common variant and 10 C77G heterozygous individuals. Differences between C77G homozygous and C77C common variant individuals were analysed using the Mann-Whitney test: * p= 0.0004, + p=0.04.

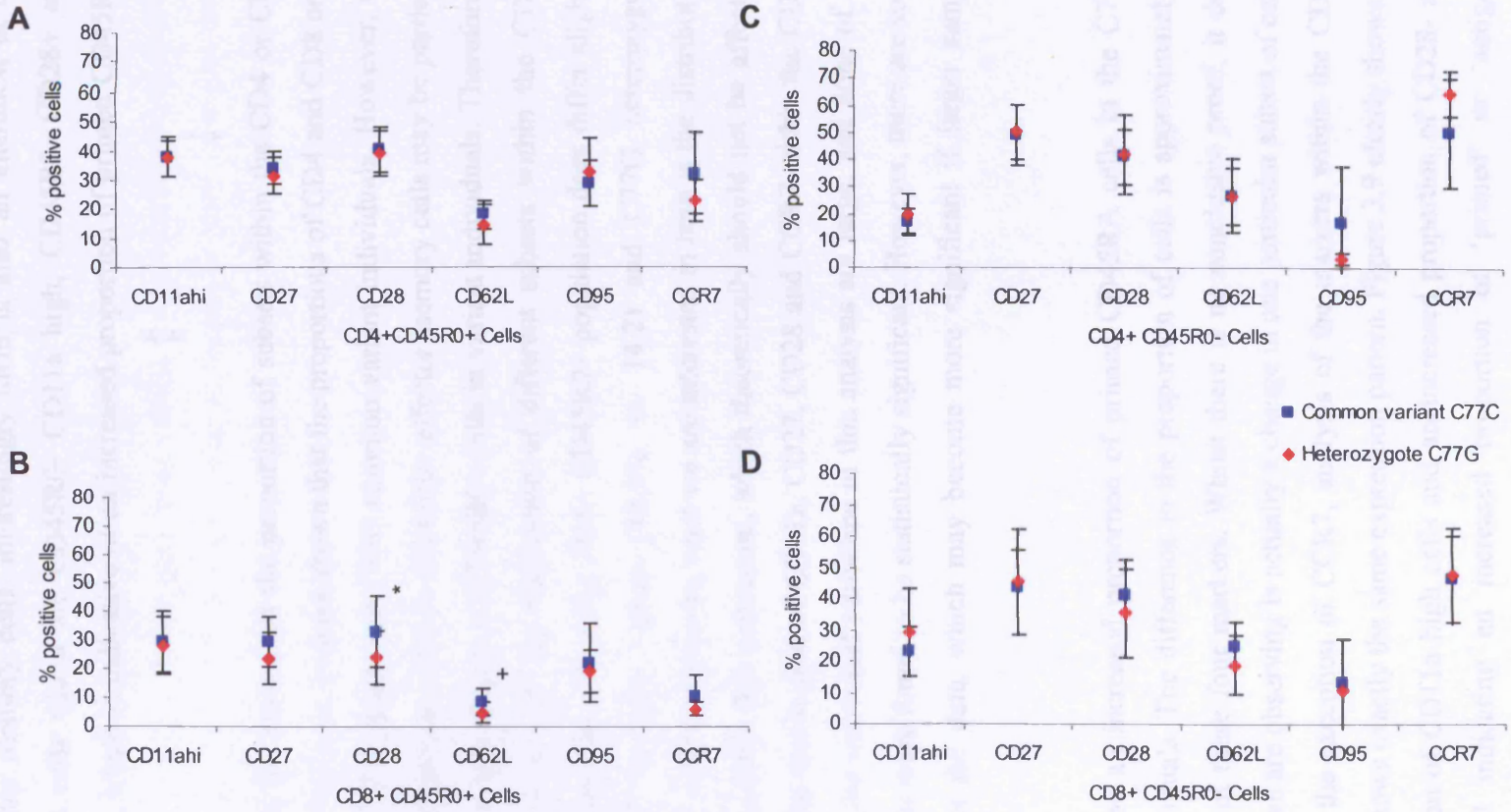



**Figure 3.7 Phenotype of CD4 and CD8 cell subsets in common variant C77C and heterozygous C77G individuals.** Proportions of (A) CD4 and (B) CD8 T cells that express CD45R0 and CD11a, CD27, CD28, CD62L, CD95 or CCR7. Proportions of (C) CD4 and (D) CD8 T cells which are CD45R0 negative and express CD11a, CD27, CD28, CD62L, CD95 or CCR7. Data shows means and standard deviation of 10 C77C common variant and 10 C77G heterozygous individuals. Differences between C77G heterozygous and C77C common variant individuals were analysed using the Mann-Whitney test: * P=0.045, + P= 0.049

decrease in the proportion of CD8 cells which are CD45R0+ CD27+, CD45R0+ CD28+, CD45R0+ CD62L+ and CD45R0+ CCR7+. This suggests an increased proportion of effector memory cells. Interestingly there is also an alteration in the proportion of CD8 cells which are CD45R0- CD11a high, CD45R0- CD28+ and CD45R0- CD62L+, which is indicative of an increased proportion of primed CD45RA effector cells.

This analysis has so far determined the proportion of subsets within the CD4 or CD8 populations. This is valid, as we have shown that the proportions of CD4 and CD8 cells are equivalent in C77G variant and C77C common variant individuals. However, the differences in the proportion of CD8+ CD45R0+ effector memory cells may be partially caused by the altered proportions of CD45R0+ cells in variant individuals. Therefore it is also necessary to look at the distribution of different subsets within the CD8+ CD45R0+ population. The size of the CD8+ CD45R0+ population does differ slightly between the C77C common variant (42.5% +/- 14.2) and C77G heterozygous individuals (32.8% +/- 8.5). However, what we are interested in here is the distribution of different subsets within this population, which theoretically should not be affected. Figure 3.8 shows the distribution of CD11a, CD27, CD28 and CCR7 within the CD8+ CD45R0+ subset. The individual variations in this analysis are large, and none of the observed differences were found to be statistically significant. However, there are some consistent trends in the data, which may become more significant if larger sample groups can be used.

There appears to be an increased proportion of primed CD45RA cells in the C77G heterozygous individuals. The difference in the proportion of cells is approximately a 10% shift in each of these four markers. Whilst there is no conclusive proof, it does suggest that what we are observing is actually a change in one particular subset of cells. Remarkably, with the exception of CCR7, analysis of the markers within the CD8+ CD45R0- subset shows exactly the same expression pattern. Figure 3.9 clearly shows an increased proportion of CD11a high cells and an increased proportion of CD28- and CD62L- cells, all indicating an increased proportion of 'primed' or 'antigen-experienced' cells. If anything this bias towards the primed population is slightly larger than the shift from central to effector memory observed in the CD8+ CD45R0+ subset, with a shift of around 15%. It is possible that this is a population of CD45RA 'revertant'

cells, stable populations of virus specific CD45RA+ cells which are CD11a high and CCR7 low and have shorter telomeres than 'naïve' CD45RA+ cells (Warren and Skipsey, 1991; Faint *et al.*, 2001; Kuijpers *et al.*, 2003).

Although usually a good indicator, phenotype does not necessarily correlate with function of cells. Functional studies need to be carried out to establish whether these primed CD45RA+ cells are true memory cells, or indeed if they have the same functional abilities of equivalent subsets of cells from C77C common variant individuals. Further studies could include looking at responses to antigen, ability to produce cytokines, clonality or telomere length of T cells.

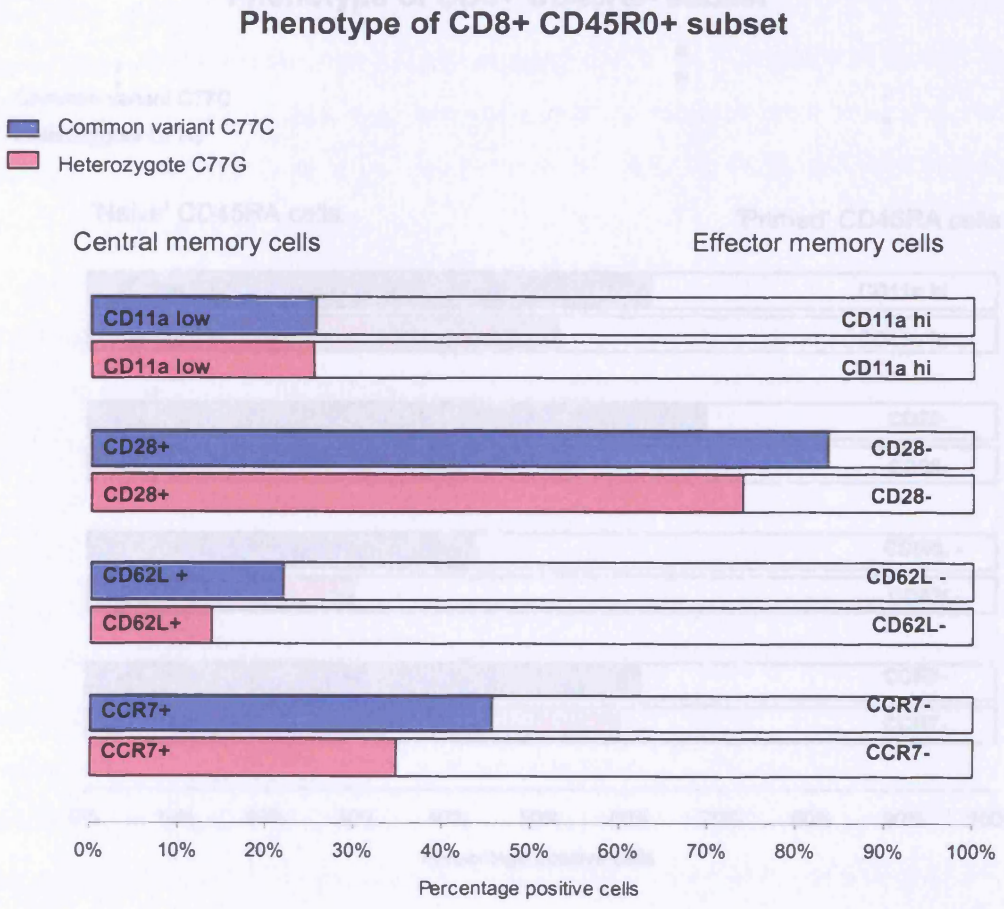



**Figure 3.8 Phenotype of CD8+ CD45R0+ subset of cells in common variant C77C and heterozygous C77G individuals.** PBMC were gated on CD8+ CD45R0+ cells and percentage of cells that express CD11a, CD28, CD62L, and CCR7 was determined. Data shows mean of 10 C77C common variant and 10 C77G heterozygous individuals.

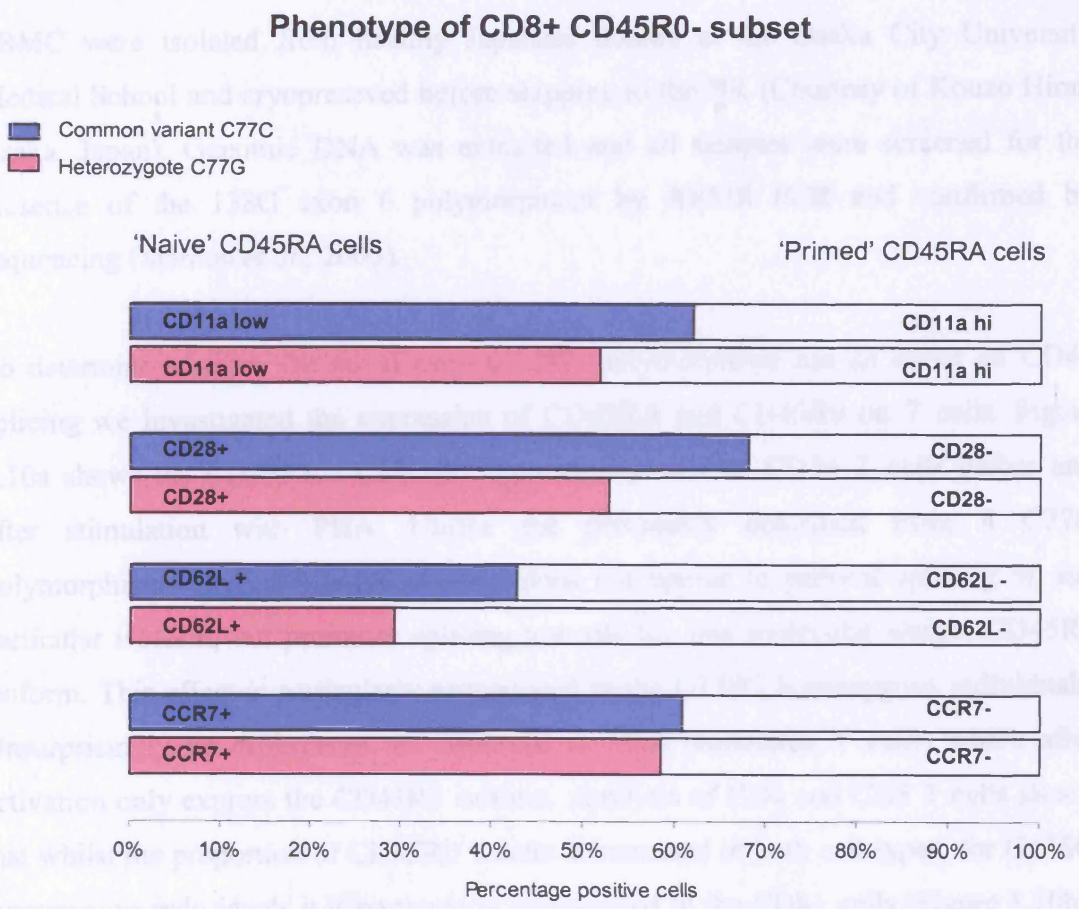



**Figure 3.9 Phenotype of CD8+ CD45R0- subset of cells in common variant C77C and heterozygous C77G individuals.** PBMC were gated on CD8+ CD45R0- cells and percentage of cells that express CD11a, CD28, CD62L, and CCR7 was determined. Data shows mean of 10 C77C common variant and 10 C77G heterozygous individuals.

### 3.3.2 The exon 6 A138G polymorphism.

#### 3.3.2.1 Variant CD45 splicing

PBMC were isolated from healthy Japanese donors at the Osaka City University Medical School and cryopreseved before shipping to the UK (Courtesy of Kouzo Hirai, Osaka, Japan). Genomic DNA was extracted and all samples were screened for the presence of the 138G exon 6 polymorphism by ARMS PCR and confirmed by sequencing (Stanton *et al.,* 2003).

To determine whether the novel exon 6 138G polymorphism has an effect on CD45 splicing we investigated the expression of CD45RA and CD45R0 on T cells. Figure 3.10a shows the CD45RA / CD45R0 expression profile of CD3+ T cells before and after stimulation with PHA. Unlike the previously described exon 4 C77G polymorphism, the 138G polymorphism does not appear to prevent splicing of any particular isoform, but promotes splicing towards the low molecular weight CD45R0 isoform. This effect is particularly pronounced in the G138G homozygous individuals. Unsurprisingly no differences are observed in PHA stimulated T cells, which after activation only express the CD45R0 isoform. Analysis of CD4 and CD8 T cells shows that whilst the proportion of CD45R0 + cells is increased in both cell types, for G138G homozygous individuals it is particularly pronounced in the CD8+ cells (Figure 3.10b). The effects of the 138G polymorphism on CD45 splicing will be investigated further using minigene constructs in Chapter 5.

#### 3.3.2.2 Cell populations

To establish whether the 138G polymorphism has any affect on the proportions of different cell types, PBMC from healthy A138A common variant and G138G homozygous individuals were stained with antibodies against CD3, CD4, CD8, CD14, CD19 and CD56. Figure 3.11a shows the proportion of the different cell types (mean of four individuals per group). Although there is some individual variation, there are no significant differences ($p>0.1$) in the proportions of T cells (CD3+, CD4+ or CD8+), B cells (CD19+), monocytes (CD14+) or NK cells (CD56+).

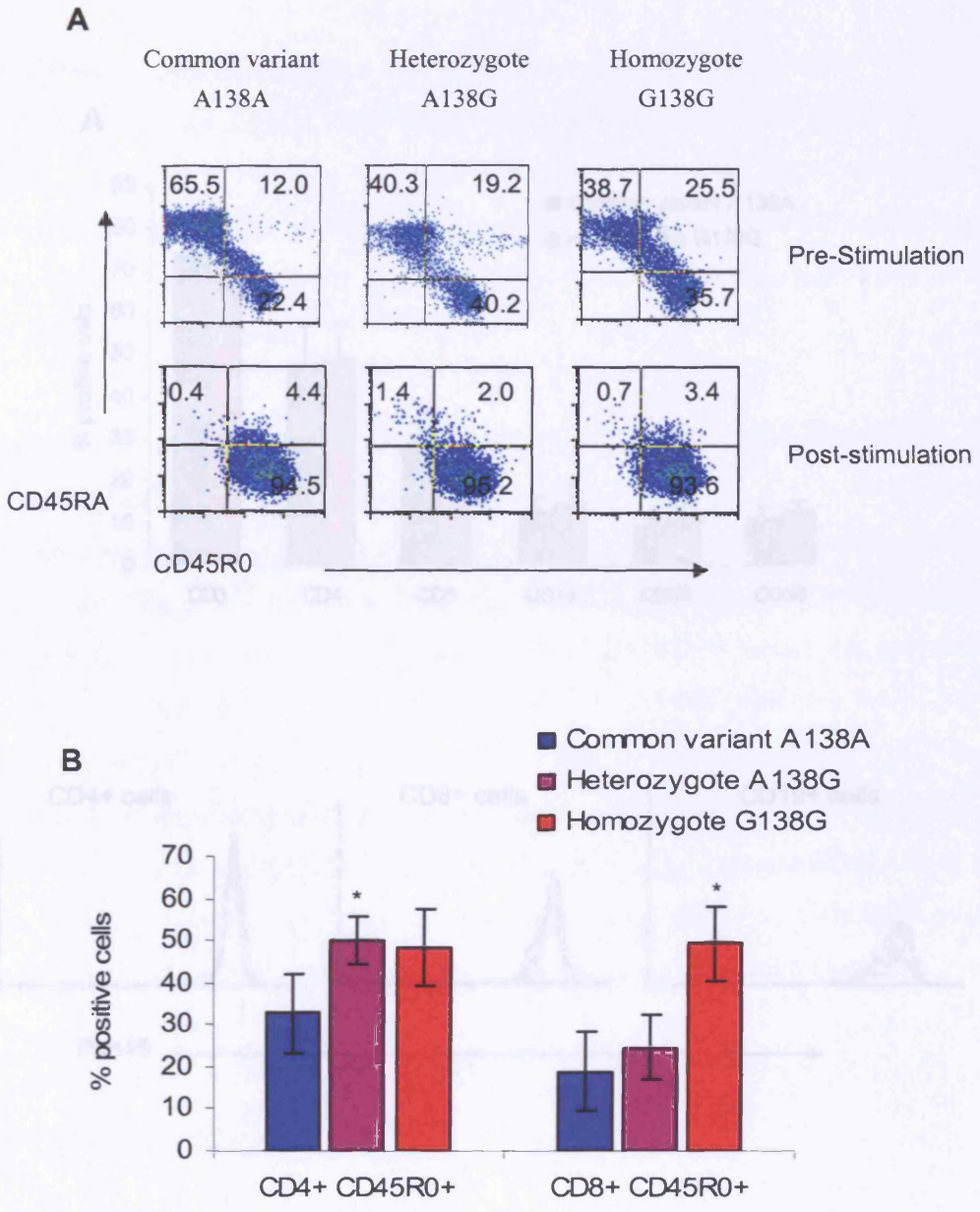



**Figure 3.10 Flow cytometric analysis of variant CD45 splicing.** A. PMBCs were stained with CD3-APC, CD45R0-PE and CD45RA-FITC antibodies pre and post-stimulation with 10μg/ml PHA for 10 days. Analysis was performed on CD3 gated T cells. Variant CD45 expression is characterised by the increased number of cells expressing CD45R0 before stimulation. Normal and variant CD45 cells show loss of CD45RA and gain of CD45R0 expression upon activation. B. PBMC were stained with CD4 or CD8 and CD45R0. Both heterozygous A138G and homozygous G138G individuals have increased percentages of CD45R0+ cells. Data shows mean and standard deviation for 4 individuals per group. * p= 0.030. Differences between A138G or G138G and A138A individuals were analysed using the Mann-Whitney test.

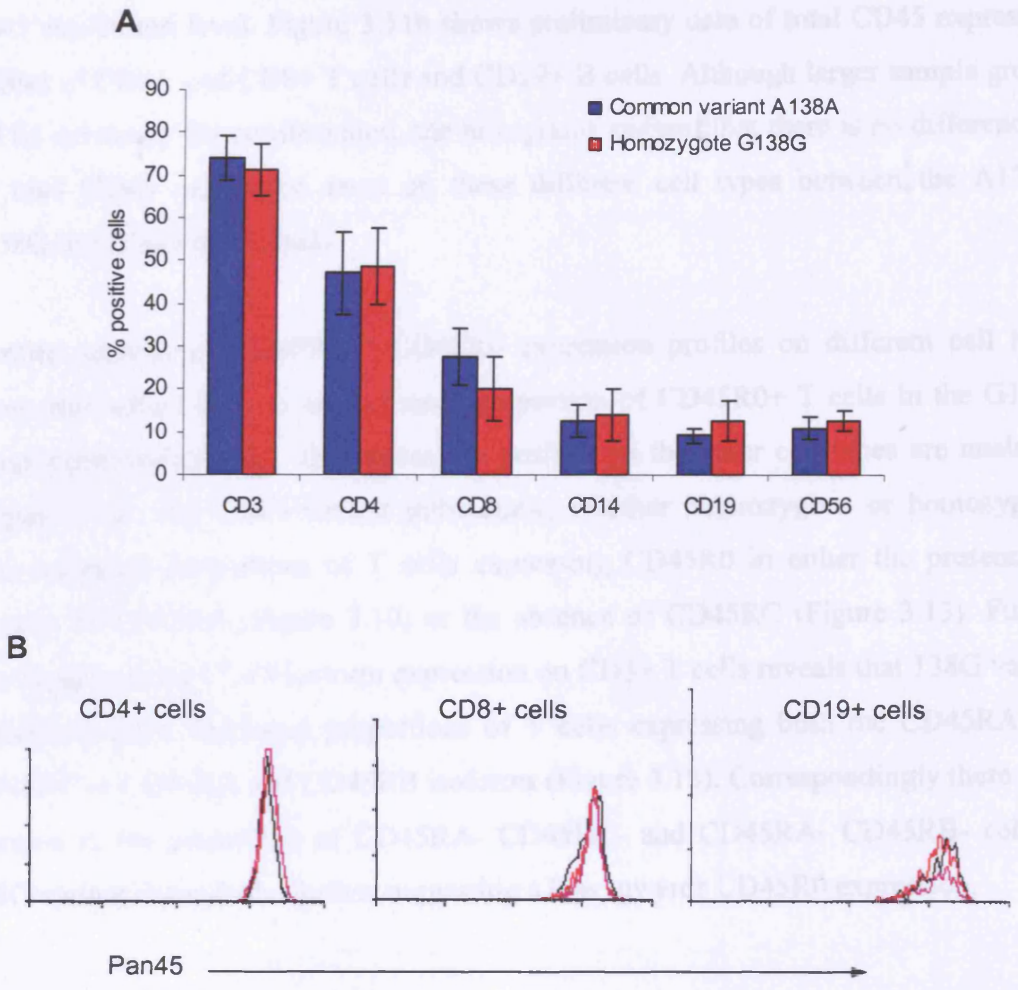



**Figure 3.11 (A). Percentage of different populations of cells in PBMC from common variant A138A and homozygous G138G individuals.** Mean of four individuals per group, error bars give standard deviations. (B). Total CD45 expression profiles on CD4+, CD8+ and CD19+ cells. PBMC were stained with Pan45-PE (a monoclonal antibody against an epitope present on all CD45 isoforms) and CD4, CD8 or CD19. Histograms show one representative individual from 2 A138A common variant individuals (black line), 2 A138G heterozygous individuals (purple line) and 2 G138G homozygous individuals (red line).

### 3.3.2.3 CD45 isoform expression

It is important to establish whether the 138G polymorphism has an effect on the total CD45 expression level. Figure 3.11b shows preliminary data of total CD45 expression profiles of CD4+ and CD8+ T cells and CD19+ B cells. Although larger sample groups will be necessary for confirmation, the histograms suggest that there is no difference in the total CD45 expression level on these different cell types between the A138A, A138G or G138G individuals.

Further analysis of CD45RA / CD45R0 expression profiles on different cell types shows that whilst there is an increased proportion of CD45R0+ T cells in the G138G homozygous individuals, the expression profiles on the other cell types are unaltered (Figure 3.12). The 138G variant individuals, whether heterozygous or homozygous, have increased proportions of T cells expressing CD45R0 in either the presence or absence of CD45RA (Figure 3.10) or the absence of CD45RC (Figure 3.13). Further investigations into CD45 isoform expression on CD3+ T cells reveals that 138G variant individuals have decreased proportions of T cells expressing both the CD45RA and CD45RC or CD45RA and CD45RB isoforms (Figure 3.13). Correspondingly there is an increase in the proportion of CD45RA- CD45RC- and CD45RA- CD45RB- cells in 138G variant individuals, further suggesting a bias towards CD45R0 expression.

### 3.3.2.4 T cell phenotype

Although we have shown that the 138G variant allele has a quantitative effect on CD45 splicing, with an increase in the proportion of CD45R0+ T cells, little is known about the effects of this on other aspects of T cell phenotype. Whilst phenotypic characterisation is useful for defining populations of cells, particularly for identifying naïve / memory cell populations, none of the markers used are completely accurate in representation of cell function and stage of differentiation. Establishing that there are an increased proportion of CD45R0+ cells does not necessarily mean that there is a corresponding increase in true memory cells (i.e. members of expanded clones).

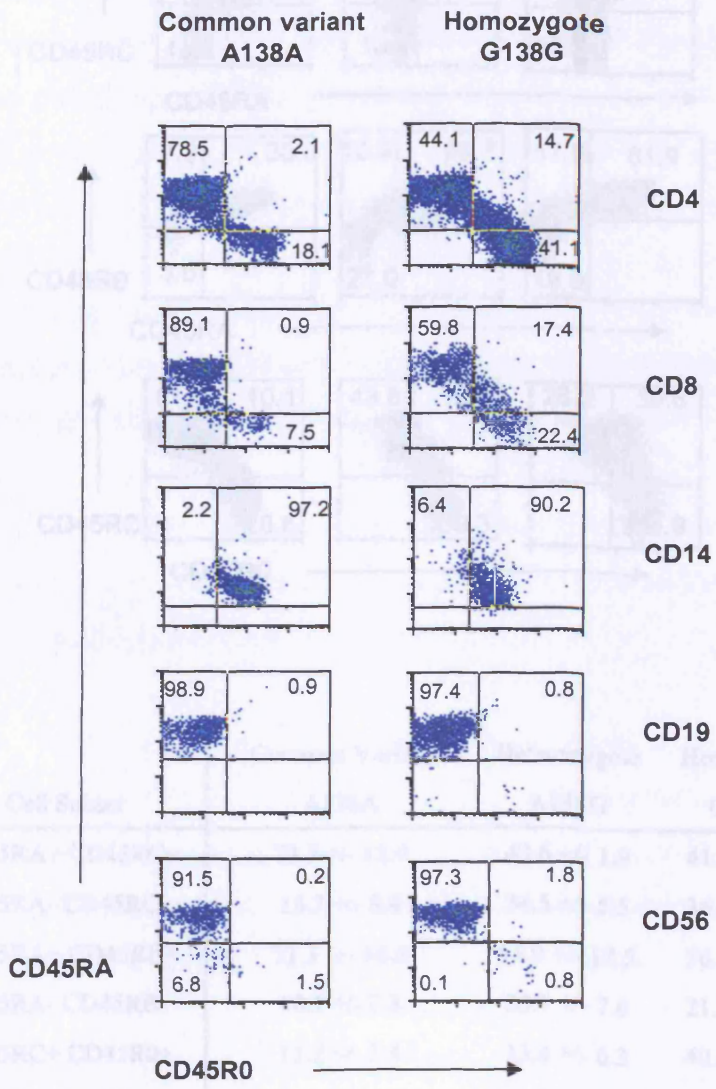
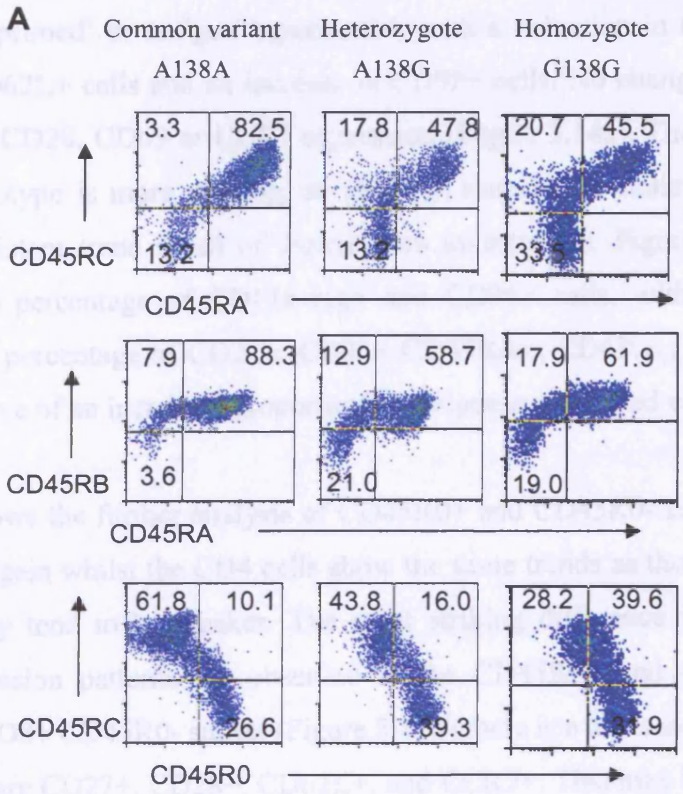



**Figure 3.12 CD45 isoform expression on different cell types.** PBMC were stained with a range of cell type specific antibodies together with isoform specific CD45R0 and CD45RA antibodies. Profiles are shown gated on a different cell types. Examples are representative of similar analyses on 6 A138A common variant individuals and 4 G138G homozygous individuals.

A




B

| Cell Subset | Common Variant A138A | Heterozygote A138G | Homozygote G138G |
|---|---|---|---|
| CD45RA+ CD45RC+ | 73.7 +/- 12.0 | 49.6 +/- 1.9 | 41.5 +/- 5.5 |
| CD45RA- CD45RC- | 18.7 +/- 8.4 | 34.5 +/- 5.5 | 36.0 +/- 3.7 |
| CD45RA+ CD45RB+ | 71.3 +/- 14.1 | 55.9 +/- 12.5 | 56.3 +/- 5.5 |
| CD45RA- CD45RB- | 10.1 +/- 7.8 | 20.7 +/- 7.6 | 21.8 +/- 2.1 |
| CD45RC+ CD45R0+ | 11.2 +/- 5.4 | 13.4 +/- 6.3 | 40.6 +/- 5.7 |
| CD45RC- CD45R0+ | 20.1 +/- 6.6 | 32.0 +/- 6.4 | 36.1 +/- 5.0 |

**Figure 3.13 Expression of CD45 isoforms on CD3+ T cells.** (A) PBMC were stained with isoform specific antibodies against CD45RA, CD45RB, CD45RC and CD45R0. Analysis was performed on CD3+ gated cells. Examples representative of similar analysis of four individuals per group. (B) CD45 isoform expression expressed as a percentage of CD3+ T cells. Mean and standard deviation of four A138A common variant, four A138G heterozygous and four G138G homozygous individuals.

Initial phenotypic analysis shows that the phenotype of CD4 cells from 138G variant individuals is 'primed' or antigen experienced, with a reduction in the proportions of CD27+ and CD62L+ cells and an increase in CD95+ cells. No change was detected in CD11a, CD25, CD28, CD69 or CCR7 expression (Figure 3.14a). The difference in the CD8 cell phenotype is more striking, as although not always statistically significant, there is a consistent trend in all of the markers investigated. Figure 3.14b shows an increase in the percentage of CD11a high and CD95+ cells, with a corresponding decrease in the percentage of CD27+, CD28+ CD45RA+, CD62L+, and CCR7+ cells. This is suggestive of an increased proportion of antigen experienced effector cells.

Figure 3.15 shows the further analysis of CD45R0+ and CD45R0- subsets of CD4 and CD8 T cells. Again whilst the CD4 cells show the same trends as those observed in the CD8 cells, they tend to be weaker. The most striking difference is that completely different expression patterns are observed in the CD45R0+ and CD45R0- subsets. Amongst the CD8+ CD45R0- subset (Figure 3.15d) there is a decrease in the percentage of cells which are CD27+, CD28+, CD62L+, and CCR7+. This may be indicative of an increased proportion of primed CD45RA cells.

In the CD8+ CD45R0+ subset, G138G homozygous individuals appear to have increased proportions of CD27+, CD28+ and CCR7+ cells (Figure 3.15b). This would suggest the presence of increased proportions of resting or central memory cells. To further establish the relative proportions of antigen experienced cells within each subset, the phenotype of cells within the CD8+ CD45R0+ subset was determined.

Whilst not statistically significant on this limited sample set, consistent differences can be observed in both the CD8+ CD45R0+ and CD8+ CD45R0- subsets from G138G homozygous compared to A138A common variant individuals. Figure 3.16 shows that an increased proportion of the CD8+ CD45R0+ subset are CD11a low, CD27+, CD28+ and CCR7+ in the G138G variant compared to A138A common variant individuals. This may be indicative of a central memory phenotype. However, caution must be taken when interpreting this subset, as the CD45R0+ subset also includes double positive CD45RA+ CD45R0+ cells. In this instance the CD8+ cells from A138G homozygous individuals contains an increased proportion of CD45R0+ CD45RA+ cells (22.5% +/- 5.1) compared to the A138A common variant individuals (12.7% +/- 10.2). This double

positive population is a transient population, with a variable phenotype, which when included in the analysis of the CD45R0 subset may have an effect on the observed phenotype.

When looking in further detail at the CD8+ CD45R0- subset it can be seen that, with some individual variation, the G138G variant individuals tend to have an increased proportion of CD11a high, CD28- and CCR7- cells compared to A138A common variant individuals (Figure 3.17). It is also worth noting that the difference in each of these markers is 10-15%, suggesting that rather than a general increase in the proportion of primed effector cells, there may be an increase in a particular subset of antigen experienced cells.

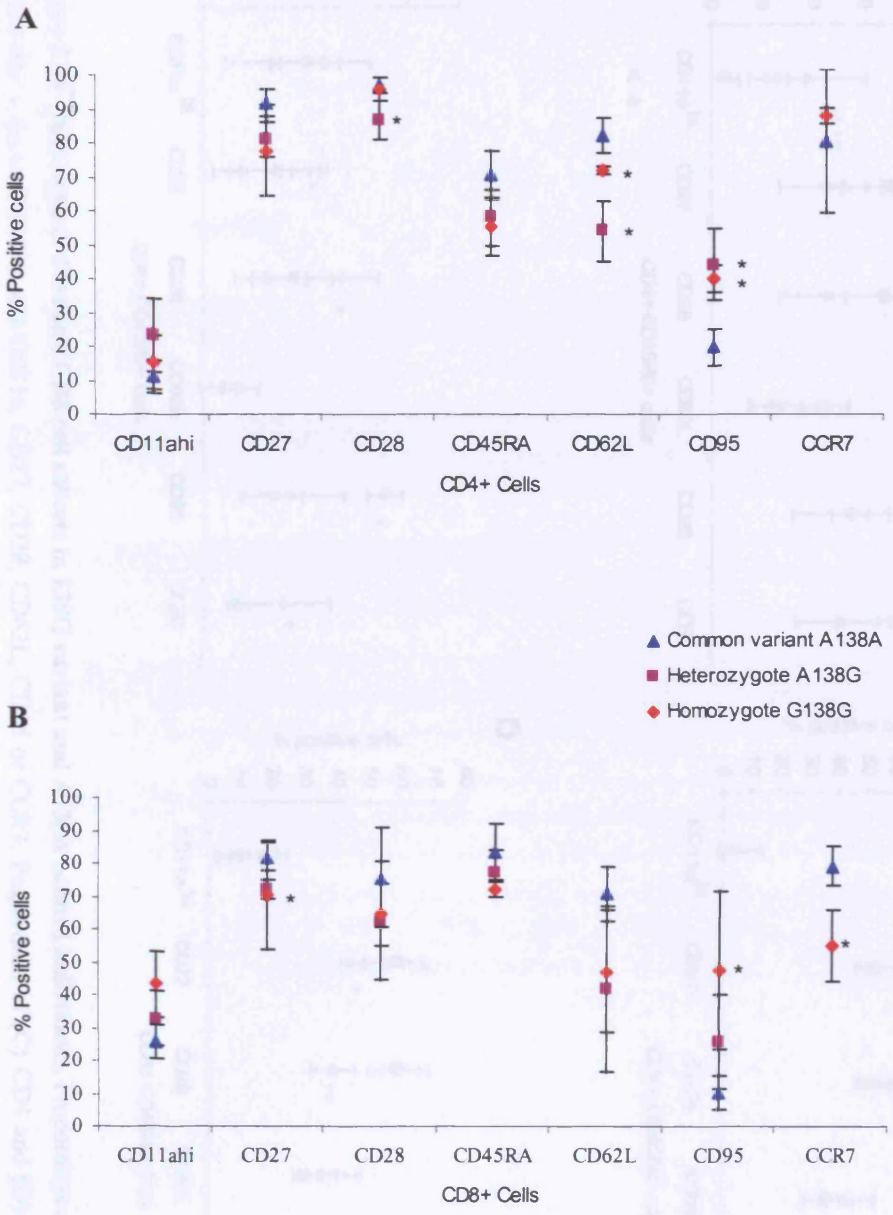



**Figure 3.14 Phenotype of CD4 and CD8 cells in 138G variant and A138A control individuals.** PBMC were stained with CD4 or CD8 and CD11a, CD27, CD28, CD45RA, CD62L, CD95 and CCR7. Proportions of (A) CD4 and (B) CD8 T cells expressing different markers. Data shows means and standard deviation of 6 A138A common variant and 4 A138G heterozygous and 4 G138G homozygous individuals. Differences between G138G or A138G individuals and A138A controls were analysed using the Mann-Whitney test: * $p < 0.030$.

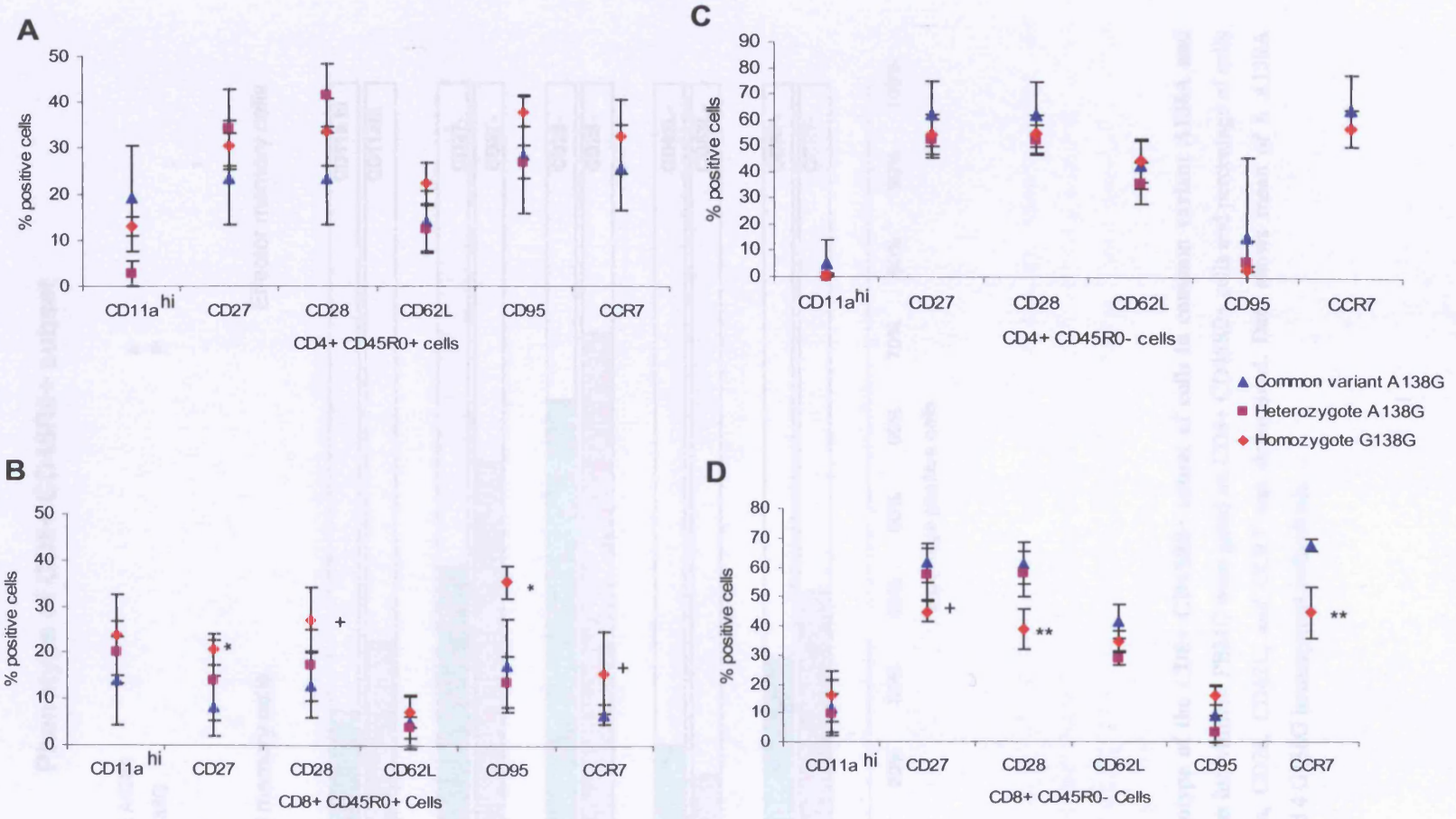



**Figure 3.15 Phenotype of CD4 and CD8 cell subsets in 138G variant and A138A control individuals.** Proportions of (A) CD4 and (B) CD8 T cells that express CD45R0 and CD11a, CD27, CD28, CD62L, CD95 or CCR7. Proportions of (C) CD4 and (D) CD8 T cells which are CD45R0 negative and express CD11ahi, CD27, CD28, CD62L, CD95 or CCR7. Data shows means and standard deviation of 6 A138A common variant and 4 A138G heterozygous and 4 G138G homozygous individuals. Differences between G138G or A138G individuals and A138A controls were analysed using the Mann-Whitney test: **p=0.014, * p=0.025, + p= 0.043.

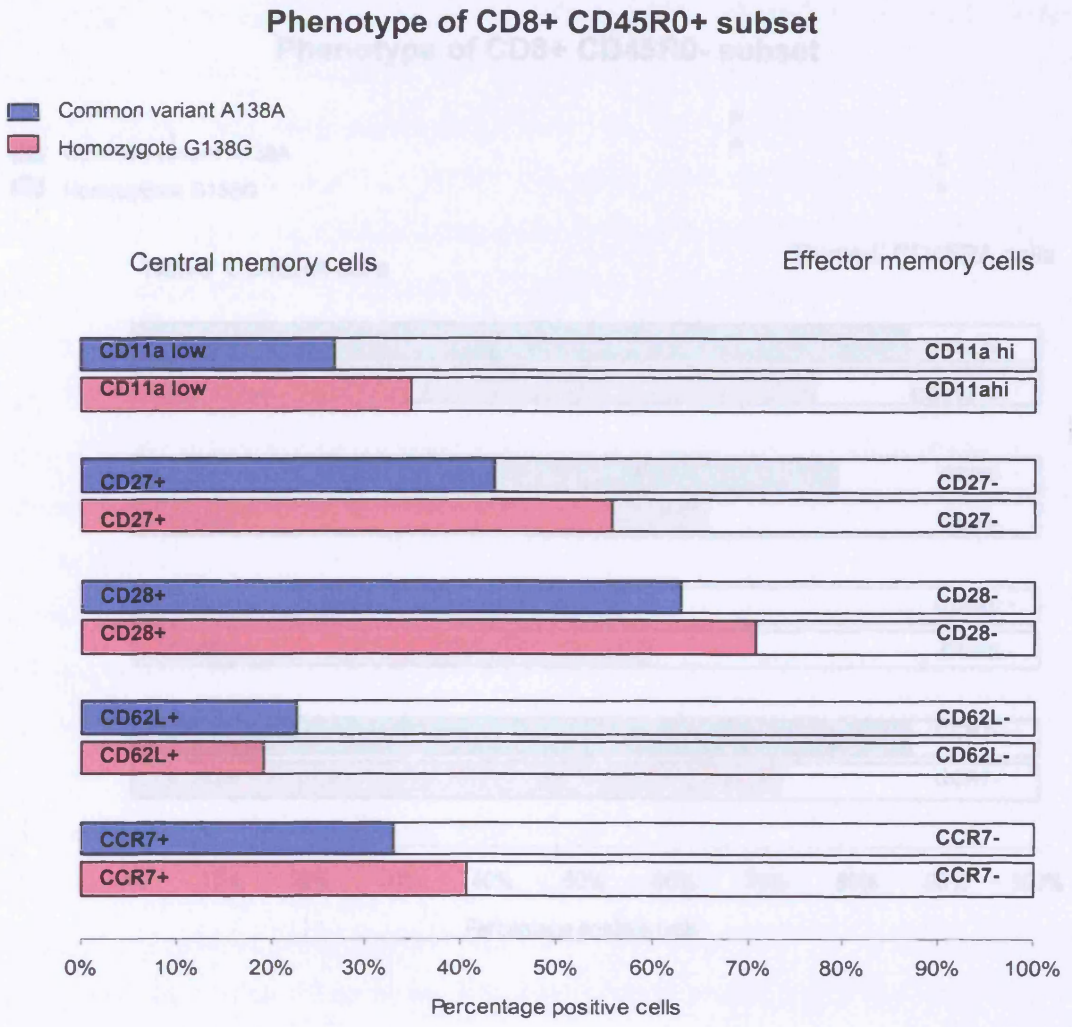



**Figure 3.16 Phenotype of the CD8+ CD45R0+ subset of cells in common variant A138A and G138G homozygous individuals.** PBMC were gated on CD8+ CD45R0+ cells and percentage of cells that express CD11a, CD28, CD62L, and CCR7 was determined. Data shows mean of 6 A138A common variant and 4 G138G homozygous individuals.

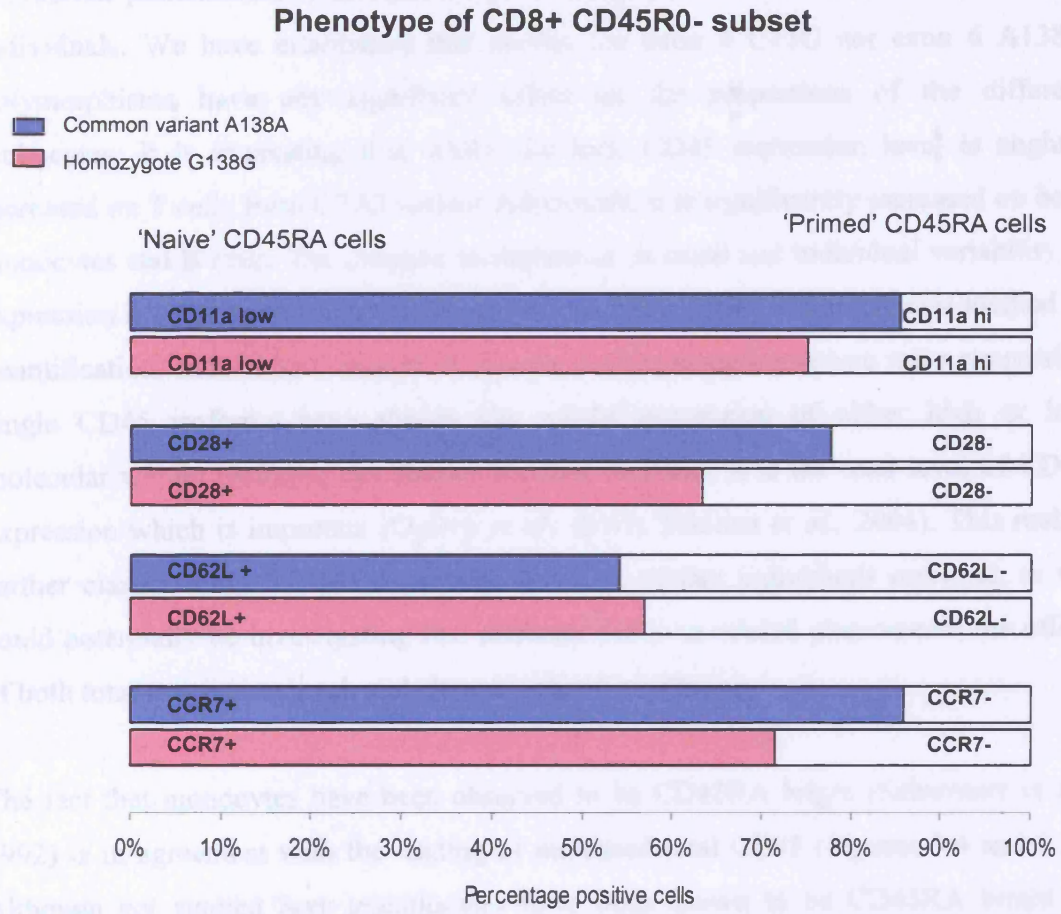



**Figure 3.17 Phenotype of CD8+ CD45R0- subset of cells in common variant A138A and homozygous G138G individuals**. PBMC were gated on CD8+ CD45R0- cells and percentage of cells that express CD11a, CD28, CD62L, and CCR7 was determined. Data shows mean of 6 A138A common variant and 4 G138G homozygous individuals.

## 3.4 Discussion

The purpose of the work discussed in this chapter has been to define the CD45 expression patterns and T cell phenotype of exon 4 C77G and exon 6 138G variant individuals. We have established that neither the exon 4 C77G nor exon 6 A138G polymorphisms have any significant effect on the proportions of the different leukocytes. It is interesting that whilst the total CD45 expression level is slightly increased on T cells from C77G variant individuals, it is significantly increased on both monocytes and B cells. The increase in expression is small and individual variability in expression is large, so this analysis should be confirmed using a more precise method of quantification, such as real time PCR. Recent studies using transgenic mice expressing single CD45 isoforms have shown that whilst expression of either high or low molecular weight isoforms can restore immune function, it is the total level of CD45 expression which is important (Ogilvy *et al.,* 2003; Tchilian *et al.,* 2004). This makes further clarification of CD45 expression level on variant individuals essential, as we could potentially be investigating two different but inter-related phenomena; the effect of both total expression level, and altered isoform expression.

The fact that monocytes have been observed to be CD45RA bright (Schwinzer et al., 1992) is in agreement with the finding of increased total CD45 (Figures 3.4 and 3.5). Although not studied here granulocytes have been shown to be CD45RA bright in C77G individuals (Schwinzer et al., 1992) so it could be postulated that they may also be found to have increased CD45 expression in C77G variant individuals. The activation state of B cells and monocytes in C77G variant individuals has yet to be established and it is possible that variant CD45 isoform expression does have an effect on cell phenotype. This would not have necessarily been expected, particularly in the case of B cells which in unstimulated PBMC are all found to be single CD45RA+. One of the many areas for further investigation in CD45 variant individuals is whether there is any difference in the phenotype or function of their peripheral B cells.

The frequency of the C77G polymorphism is low, but its presence in the healthy population suggests it is unlikely to have a dramatic effect on any of the cells types. Certainly from our observations on T cells the effects of variant isoform expression appear to be relatively subtle and more involved with altering the proportions of

subpopulations of cells. In accordance with previous studies, we have shown that individuals heterozygous for the C77G allele have a variant pattern of CD45RA / CD45R0 isoform expression on their T cells. T cells from variant individuals fail to express the single CD45R0 isoform, even after stimulation with PHA for 10 days.

The CD4+ T cells from variant individuals have a similar phenotype to that of C77C common variant individuals, both in the CD45R0+ and CD45R0- subsets. It is interesting that the CD8 T cells are more affected, a phenomenon which will be discussed further in terms of functional responses in later chapters. Despite having the same proportion of CD8 cells, the proportion of primed effector cells appears to be increased in the C77G variant individuals. This is found both in terms of the proportion of CD8 cells and also the proportions of the CD45R0+ and CD45R0- subsets within the CD8 population. However, the most pronounced effect is the increased proportion of primed CD8+ CD45RA+ cells in C77G variant individuals. As we have demonstrated that the percentage of the different cell types is equivalent in C77G variant and C77C control individuals, assuming that variant individuals do have the same total lymphocyte counts, it can be postulated that this alteration in the proportion of primed CD8+ CD45RA+ cells will equate to an increase in the absolute number of these cells in C77G variant individuals.

Previously a subset of CD8+ CD45RA+ T cells have been described with a distinct phenotype, which after priming 'revert' from expressing CD45R0 to expressing CD45RA. These were found to be a stable population of virus specific cells, with a CD11a high, CCR7 low phenotype and shorter telomeres than CD45RA+ CD11a low cells (Warren and Skipsey, 1991; Wills *et al.*, 1999; Faint *et al.*, 2001; Dunne *et al.*, 2002; Kuijpers *et al.*, 2003). The existence of CD4+ CD45RA+ revertant cells is more controversial. Whilst memory cells have been shown to be present in the CD4+ CD45RA+ population (Richards *et al.,* 1997) reversion from CD45R0 to CD45RA expression has not been well documented in CD4 cells. This may in part explain why in the C77G variant individuals, primed cells are primarily observed in the CD8+ CD45RA+ and not CD4+ CD45RA+ cell subsets.

The increased proportion of CD8+ CD45RA+ 'primed' effector type cells observed in the C77G variant individuals may be a 'revertant' population. It is plausible that the

block in completely switching to single CD45R0 expression in some way increases their ability to 'revert' to CD45RA expression. Of course the phenotypic identification of a 'primed' population CD8+ CD45RA+ cells is not conclusive proof that these are revertant cells. To prove this, detailed analysis of the naïve and primed CD8+CD45RA+ cells would be required, which may include looking at telomere length to determine number of cell divisions or proliferative and cytokine responses. The problem with such experiments is that the better defined the subset of cells is, the smaller it is, requiring large amounts of starting material and making them unfeasible in the present study.

If this population is identified as a 'revertant' population it does pose an interesting question. In Chapter 1 we discussed the possible associations of the C77G polymorphism with disease. The most striking associations are with viral infection, particularly with susceptibility to and failure to clear Hepatitis C infection (E.Tchilian, Personal communication). As CD45RA 'revertant' cells are usually virus specific, often correlating with CMV seropositivity (Kuijpers *et al.*, 2003) the question is whether the abnormal T cell phenotype observed in C77G variant individuals has a role in the susceptibility to and inability to clear viral infections, or is merely a consequence of it. It should also be remembered that the proportions of CD8+ CD45RA+ effector cells increases with age (Hong *et al.*, 2004), which will also correlate with other factors such as CMV seropositivity, as the likely hood of infection also increase with age. All of the individuals in this study were middle aged (between 23 and 58 years old), with variant and control individuals being age matched as closely as possible. Therefore any effects of age on the proportions of different cell subsets should be equivalent in each group, but may be an important factor in designing further studies.

The second part of this chapter involves the phenotypic characterisation of T cells from individuals with the exon 6 138G variant allele. As with the previously described C77G polymorphism, we have shown that variant allele expression does not have an effect on the proportions of the different cell types in the peripheral blood, or the total CD45 expression level on T cells. Further to this we have demonstrated that the T cells from 138G variant individuals are not lacking in their expression of any of the CD45 isoforms. However, whilst there were no qualitative differences in isoform expression there is a quantitative difference in the proportion CD45R0 cells, both with and without CD45RA expression, in unstimulated T cells. Corresponding to this we have also

observed a decrease in the proportion of T cells expressing CD45RA and CD45RC or CD45RA and CD45RB. Upon stimulation with PHA all of the T cells switched to expressing low molecular weight isoforms (CD45R0 and CD45RB).

Phenotypic analysis of both CD4 and CD8 cells shows an increased proportion of antigen experienced or activated cells in 138G variant individuals. Detailed analysis of the CD45R0+ subset suggests that it contains fewer effector type cells (CD45R0+ CCR7-) in 138G variant individuals, and an increased proportion of central memory cells (CD45R0+ CCR7+). This holds whether considering the subset as a proportion of CD8+ cells or as a proportion of the CD8+ CD45R0+ cells, suggesting that it is not just an artefact of the increased number of CD45R0+ cells.

Phenotype alone can not establish whether this is a true memory subset, and further functional studies are necessary. It is possible that this increased memory phenotype subset could actually contain highly diverse naïve cells. Alternatively it could contain more diverse expanded memory clones than those in 138A control individuals, or simply larger clones than are normally observed, which may not offer any kind of advantage. There is also a question as to whether these individuals are more susceptible to disease. To date the disease association studies have shown a protective effect of the 138G variant allele against certain autoimmune and infectious diseases (as discussed in chapter 1). However if there is an increase in the memory cell repertoire, this may suggest an increased exposure to antigen, and therefore possibly an increased history of infection.

Whilst variant CD45 expression does not have an effect on the proportions of leukocytes, it does have a profound effect on the proportions of T cell subpopulations. In exon 4 C77G variant individuals there is an increased proportion and absolute number of CD8+CD45RA+ effector cells, whilst in the exon 6 138G variant individuals, there is an increased proportion of central memory cells (CD8+ CD45R0+CCR7+). The functional consequences of these phenotypic alterations will be investigated in the next chapter.

# CHAPTER 4

## Effects of variant CD45 expression on *in vitro* functional responses

### 4.1 Introduction

The phosphatase activity of the CD45 antigen is essential for efficient antigen receptor signalling, as has been demonstrated in CD45-negative cell lines (Pingel and Thomas, 1989; Weaver *et al.*, 1991) , CD45-deficient mice (Kishihara *et al.*, 1993; Byth *et al.*, 1996) and humans (Kung *et al.*, 2000; Tchilian *et al.*, 2001). Mice and humans lacking CD45 are severely immunodeficient, with few peripheral T cells and impaired T and B cell responses.

The main substrates of CD45 phosphatase activity have been shown to be members of the Src family of tyrosine kinases. CD45 can operate as both a positive and negative regulator of Src family kinases (Ostergaard *et al.*, 1989; Shiroo *et al.*, 1992). It has also been suggested that CD45 can function as a Janus kinase (JAK) phosphatase, negatively regulating cytokine receptor signalling which is involved in the differentiation, proliferation and anti-viral activity of haematopoietic cells (Irie-Sasaki *et al.*, 2001). This would suggest that differential isoform expression has a role in the regulation of cytokine production and proliferative responses after stimulation of the T cell antigen receptor.

Several groups have investigated the proliferative responses of 'naïve' CD45RA+ and 'memory' CD45R0+ T cells to various stimuli. It has been demonstrated that alloantigens and mitogenic lectins can activate both CD45R0+ and CD45RA+ cells to the same extent. It has also been demonstrated that responses to recall antigens are markedly increased in the CD45R0+ subset (Merkenschlager *et al.*, 1988; Plebanski *et al.*, 1992), in terms of both magnitude and kinetics (Young *et al.*, 1997).

Robinson *et al.*, (1993) showed an increase in proliferative response in the CD45R0+ subset after stimulation with anti-CD3 antibodies. This study measured CD3 antigen-induced calcium signals, diacylglycerol (DAG) production and protein kinase C (PKC) activation levels in both the CD45R0+ and CD45RA+ subsets. The CD3-induced rise in intracellular calcium was found to be higher in the CD45R0+ subset, as was the PKC activation level. The CD45R0+ cells were also found to have higher basal levels of DAG and PKC activity, suggesting that the signalling thresholds may be altered in these cells.

Kuiper *et al.*, (1994) investigated the different responses of CD4+ CD45R0+ and CD4+ CD45RA+ cells to co-stimulation with CD3 and CD28. This study found that even in the presence of optimal CD28 co-stimulation, naïve CD45RA+ cells are still less responsive to CD3 stimulation and required a higher level of both CD3 and CD28 cross-linking than memory cells. This supports the idea that whilst the signalling mechanisms are the same in both subsets, it is the signalling threshold that is critical for activation. It was further demonstrated by Schwinzer *et al.*, (1994) that when CD3 stimulation was combined with activation of PKC by phorbol ester, the naïve CD45RA+ cells responded equally as well as the CD45R0+ cells. They suggest that both subsets have the same potential for signalling through the TCR, and it is the co-stimulatory signals which ultimately determine the strength of the response.

These results are seemingly contradicted in a study of CD4 cells by Hall et al., (1999), in which the naïve CD45RA+ cells were found to give the largest rise in intracellular calcium and greater inositol triphosphate generation. This suggests that antigen receptors on naive cells are able to signal more effectively, especially at an early stage. This disparity could be explained by the fact that in the study by Robinson *et al.*, (1993) there were a proportion of recently activated cells in the CD45R0+ subset. These recently activated HLA-DR+ cells were excluded from the study of Hall *et al.*, (1999), which would undoubtedly have an effect on the kinetics and magnitude of the observed response. Whilst HLA-DR is not the most reliable marker for activation status and others such as CD25, CD69 or CD54 (ICAM-1) are more commonly used, these studies do show the effect of including small subsets of activated cells.

One of the major problems with comparing data from different proliferative studies is the comparison of slightly different subsets. This is dependant upon the separation protocols used, particularly whether double positive (CD45RA+CD45R0+) cells are completely excluded or are partially included in either subset. This can be further complicated, particularly in CD8+ cells by the presence of antigen experienced CD45RA+ cells, which may have altered proliferative responses compared to truly naïve cells.

A study by Sallusto *et al.*, (1999) which investigated the responses of different subsets of CD8+ T cells, defined by CCR7 and CD45RA expression, showed that both effector memory ($T_{EM}$, CCR7- CD45RA-) and central memory ($T_{CM}$, CCR7+ CD45RA-) cells respond better to stimulation with either CD3 alone, or co-stimulation with CD3 and CD28 than naïve (CCR7+ CD45RA+) cells. They also demonstrated that the effector memory cells required lower doses of stimuli and responded quicker then the central memory cells.

This shows that there are phenotypic differences within both the CD45R0 and CD45RA populations, giving differences in the proliferative potential between and within the CD45R0 and CD45RA subsets. Both C77G and 138G variant individuals have altered isoform expression on their T cells, so it could be speculated that the proliferative responses particularly to TCR stimulation may also be altered.

As previously discussed in chapter one, upon activation of naïve CD4+ cells in the presence of IL-12, IFNγ or TGFβ, the Th0 cells differentiate into Th1 cells, which can release cytokines such as IFNγ and TNFβ. An important role of IFNγ is in the activation of macrophages, increasing their antimicrobial efficiency and targeting bacterial infections. IFNγ also acts to inhibit the polarisation of Th2 cells. In the presence of IL-4, Th0 cells differentiate into a Th2 phenotype. Th2 cells can release IL-4 and IL-5 and also IL-6, 9, 10 and 13. Th2 cells can induce activation of mast cells and eosinophils. IL-4 and IL-5 can also provide T cell help to B cells to produce antibodies (Romagnani, 1992; Lehar and Bevan, 2004).

Activation of naïve CD8+ cells drives differentiation into Tc1 or Tc2 cells. Tc1 cells, often referred to cytolytic T cells (CTL) have direct cytolytic activity, release IL-2 and IFNγ, and are essential in the clearance of intracellular pathogens. Tc2 cells have a helper function similar to CD4+ cells and release cytokines including IL-4, 5, 6 and 10 (Seder *et al.*, 1992). Upon re-stimulation both subsets of CD8 cells become cytotoxic (Cerwenka *et al.*, 1998).

On stimulation with PMA and Ionomycin CD4+ cells produce more IL-2 and IL-4, whilst CD8+ cells produce more IFNγ and TNFα. CD45R0+ cells secrete greater amounts of all of these cytokines and show different kinetics to the CD45RA+ cells (Conlon *et al.*, 1995; Mascher *et al.*, 1999). However there is a subset of CD8+ CD45RA+ antigen experienced cells (CD11a high, CCR7 low) which have also been shown to be potent producers of IFNγ (Faint *et al.*, 2001). Given the complex and variable nature of cytokine production in different subsets, alterations in the proportions of effector and memory cells would have an effect on the balance of cytokines produced. Since CD45 variant individuals have altered proportions of activated and memory cells, it is possible that the cytokine profiles in these individuals are also altered.

## 4.2 Objectives

The aim of this chapter is to determine whether the altered proportions of naïve and activated T cells observed in CD45 variant individuals alter their functional responses. Proliferative responses and cytokine production to different stimuli were investigated in both C77G and 138G variant individuals.

## 4.3 Results

### 4.3.1 Proliferative responses of PBMC

#### 4.3.1.1 The exon 4 C77G variant carriers

In the previous chapter we have shown that C77G variant individuals have increased proportions of primed CD8+ CD45RA+ T cells. The only study to describe the proliferative response of T cells in C77G variant individuals was by Schwinzer and Wonigeit (1990). In their study PMBC were stimulated with CD3 or PHA for 4 days and no significant differences were found between the 3 C77G variant and 4 control individuals in response to either stimulus.

To investigate the effect of the C77G variant allele on proliferation, we stimulated PBMC from variant and control individuals with PHA, CD3 and PMA and Ionomycin, determining dose dependency and time course of response. PHA-P is a lectin isolated from the red kidney bean *Phaseolus vulgaris*, which binds glycoproteins and has a variety of uses including mitogenic stimulation of lymphocytes. PMA has an analogous structure to Diacylglycerol (DAG), which is an allosteric activator of protein kinase C (PKC) so can activate T-cells and stimulate low-level production of IL-2. PMA was used in conjunction with Ionomycin, a calcium ionophore, which extracts $Ca^{2+}$ from aqueous to organic phase, raising free intracellular calcium and thus increasing T cell activation. Anti-CD3 antibody was used as a direct stimulator of the TCR. The UCHT1-2a clone was specifically chosen, as everybody should respond to it in the soluble form, whilst due to a polymorphism in the Fc receptor this is not the case for an IgG1 or IgG2b antibody. Cells were also co-stimulated with anti-CD3 and anti-CD28 antibodies, as CD28 is a co-stimulatory molecule which with TCR stimulation can increase proliferative responses.

No differences were observed between the variant and control groups in response to stimulation with mitogen, either PHA-P or PMA and Ionomycin. Whilst PHA-P is T cell specific, PMA and Ionomycin elicits a broad response, stimulating most cell types in the culture. As this variant allele is found in healthy individuals, it is reasonable to assume that any differences in their responses would be minor, or easily compensated

for. It is plausible that by using whole PBMC cultures any small differences in a particular cell type are being masked. We therefore focused on using TCR specific stimuli, culturing PBMC with varying doses of purified CD3 (UCHT1-2a) or co-stimulating with CD3 and CD28 (15E9).

Figure 4.1a shows the kinetics of the response to 0.25μg/ml CD3 of 5 individuals in each group. The variant and control individuals have similar kinetics, with the peak response occurring at 72 hours post-stimulation. Whilst the timing of the response is the same, there is a difference in magnitude, which is most evident at 72 hours. From figure 4.1b there appears to be a significant difference in the magnitude of the response to CD3 ($p=0.04$ using a two-tailed analysis of variance, ANOVA). This effect was found to be similar at all doses ($p=0.91$). The C77G variant samples have a slightly lower response which could mean that the variant individuals are not as efficient at signalling through the CD3 receptor.

Co-stimulating cells with CD3 and CD28 has a synergistic effect, with both control and variant samples showing a higher response than with CD3 alone, but neither responding to CD28 alone. Figure 4.2a shows the response to CD3 (0.25μg/ml) and CD28 (2.5μg/ml) co-stimulation over time. The peak response observed is still at 72 hours, and the magnitude of the response appears to be identical in both groups. This is also evident in figure 4.2b, where given a reasonably large dose of CD28, there is no difference in the proliferative responses of the two groups, even at lower doses of CD3.

This might suggest that co-stimulation with CD28 has a greater effect on PBMC from C77G variant individuals than controls, as it brings a reduced response to CD3 alone, to one of the same magnitude. However, it is also possible that the effect of CD28 co-stimulation is the same on both the variant and control samples, and it is simply that the control samples are already closer to their maximum proliferative rate.

In humans, memory T cells are generally cells which have switched to express CD45R0 on their surface. As cells from C77G variant individuals are incapable of doing this, memory cells in these individuals would express both CD45R0 and CD45RA. To determine whether this differential expression confers an effect on memory cell

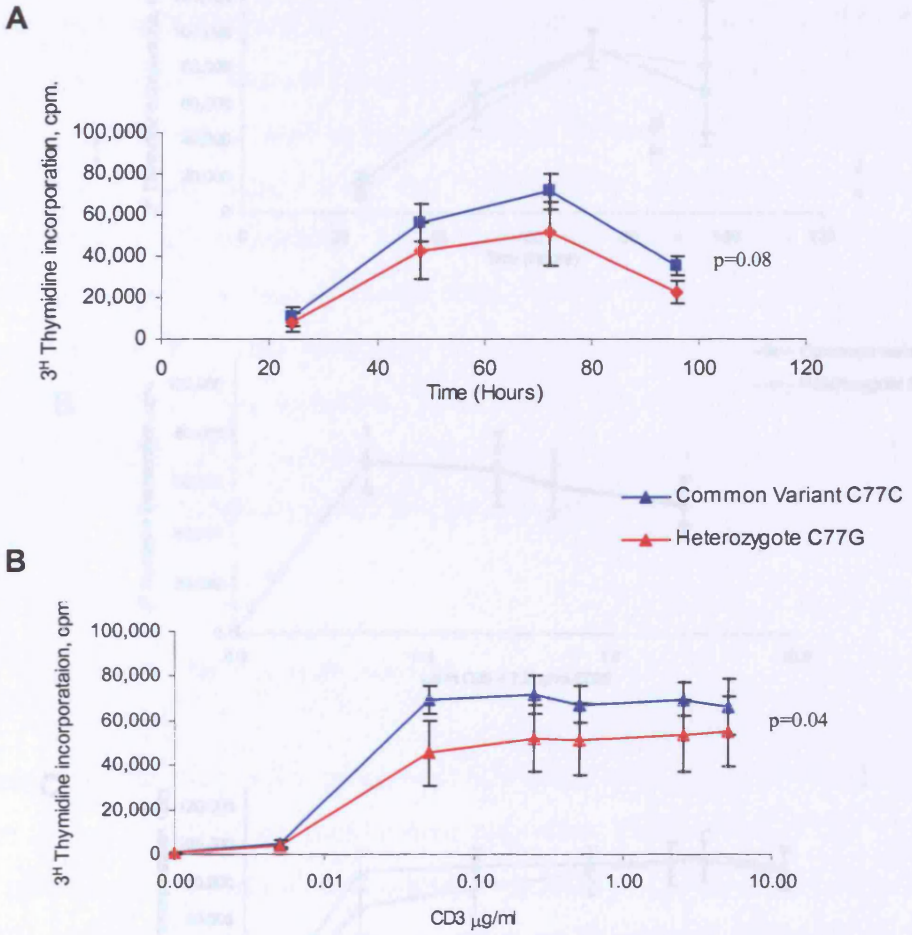



**Figure 4.1 Proliferative responses of PBMC from 5 heterozygous C77G and 5 common variant C77C individuals.** Responses were assayed by thymidine incorporation. Results given are in counts per minute (cpm) with each sample being assayed in triplicate. Each line represents the mean of 5 individuals, with the bars giving the standard error. Figures representative of 3 similar experiments. (A) Response to 0.25µg/ml of anti-CD3 (UCHT1-2a) at varying time points. Background less than 1000cpm (data not shown). Analysis of variance (ANOVA) was performed to determine the significance of the observed difference between the responses of the PBMC from C77G variant and C77C common variant individuals (p=0.08). (B) Response to varying doses of anti-CD3 (UCHT1-2a) at 72 hours. Proliferative responses of the C77C common variant individuals were higher than that of the C77G variant individuals (p=0.04).

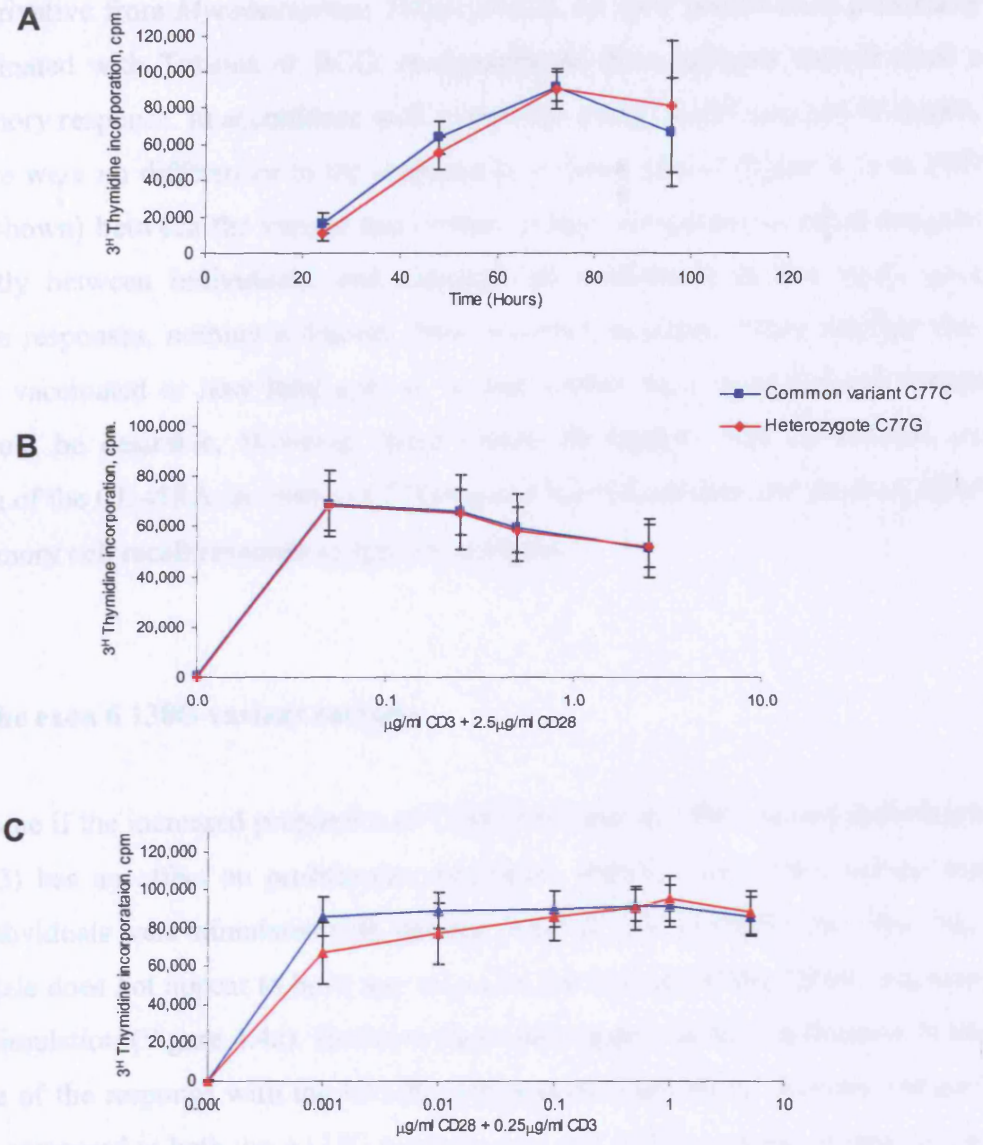



**Figure 4.2 Proliferative responses of PBMC from 5 heterozygous C77G and 5 common variant C77C individuals.** Responses were assayed by thymidine incorporation and results given in counts per minute (cpm) with each sample being assayed in triplicate. Each line on the graph represents the mean of 5 individuals, with the bars giving the standard error. Figures representative of 3 similar experiments. (A) Response to co-stimulation with 0.25µg/ml CD3 (UCHTI-2a) and 2.5 µg/ml CD28 (15E9) at varying time points. Background was less than 1500 cpm (Data not shown). (B) Response to co-stimulation with 2.5µg/ml CD28 (15E9) and varying does to CD3 (UCHT1-2a) at 72 hours. (C) Response to co-stimulation with 0.25µg/ml CD3 (UCHT1-2a) and varying doses of CD28 (15E9) at 72 hours.

function, we investigated the *in vitro* response to recall antigens. PBMC from C77G variant and control individuals were stimulated with Tetanus toxoid or PPD (Purified Protein derivative from *Mycobacterium Tuberculosis*). As most people have previously been vaccinated with Tetanus or BCG, re-exposure to these antigens should elicit a recall memory response. In accordance with a previous study (Schwinzer and Wonigeit, 1990) there were no differences in the response to Tetanus toxoid (figure 4.3) or PPD (data not shown) between the variant and control groups. Responses to recall antigens vary greatly between individuals, and although all individuals in this study gave measurable responses, nothing is known about vaccination status, either whether they have been vaccinated or how long ago, so further studies on a more defined sample group would be desirable. However, these results do suggest that the constitutive expression of the CD45RA isoform in C77G variant individuals does not have an effect on the memory cell recall response to specific antigens.

#### 4.3.1.2. The exon 6 138G variant carriers

To determine if the increased proportion of CD45R0+ cells in 138G variant individuals (Chapter 3) has an effect on proliferative responses, PBMC from 138G variant and control individuals were stimulated with varying doses of CD3 (UCHT1-2a). The 138G variant allele does not appear to have any effect on the kinetics of the PBMC response to CD3 stimulation (Figure 4.4a). However there does appear to be a difference in the magnitude of the response with the G138G homozygous individuals showing reduced responses compared to both the A138G heterozygous and A138A control groups. Given that the phenotypic changes are more extreme in the G138G homozygous individuals, it is not a surprise that the biggest differences are found in this group. Analysis of variance (ANOVA) showed significant differences between the variant groups ($p=0.001$), and that the variance was different at each time point ($p=0.019$). Further analysis for each time point was carried out using Burnnett's test for multiple comparison, and showed that the response of the G138G homozygous individuals was significantly different from both A138A control and A138G variant individuals at all time points ($p<0.05$). The magnitude of the proliferative response to CD3 is reduced in the G138G homozygous individuals compared to the A138A controls ($P<0.05$ for all doses). This suggests that the variant allele may have an effect on the efficiency of signalling

through the CD3 receptor. Due to the lack of sample material available, this data is preliminary and has yet to be repeated, preferably on a larger sample group before any conclusions can be made.

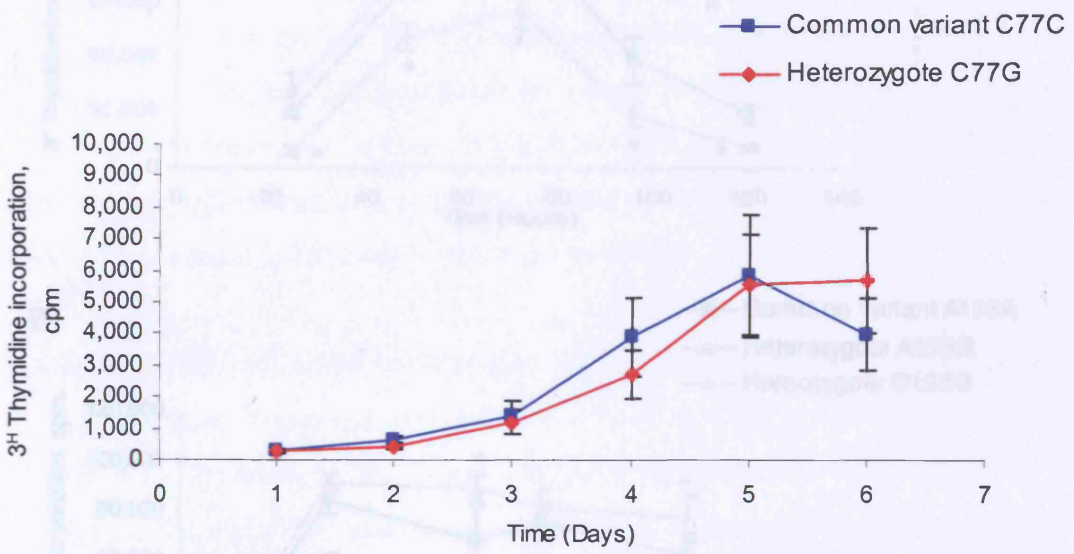



**Figure 4.3 Proliferative responses of PBMC from 10 heterozygous C77G and 10 common variant C77C individuals to 5μg/ml of Tetanus Toxoid at varying time points.** Responses were assayed by thymidine incorporation. Results given are in counts per minute (cpm) with each sample being assayed in triplicate and background subtracted. Each line on the graph represents the mean of 10 individuals, with bars giving the standard error. Figure representative of 2 similar experiments.

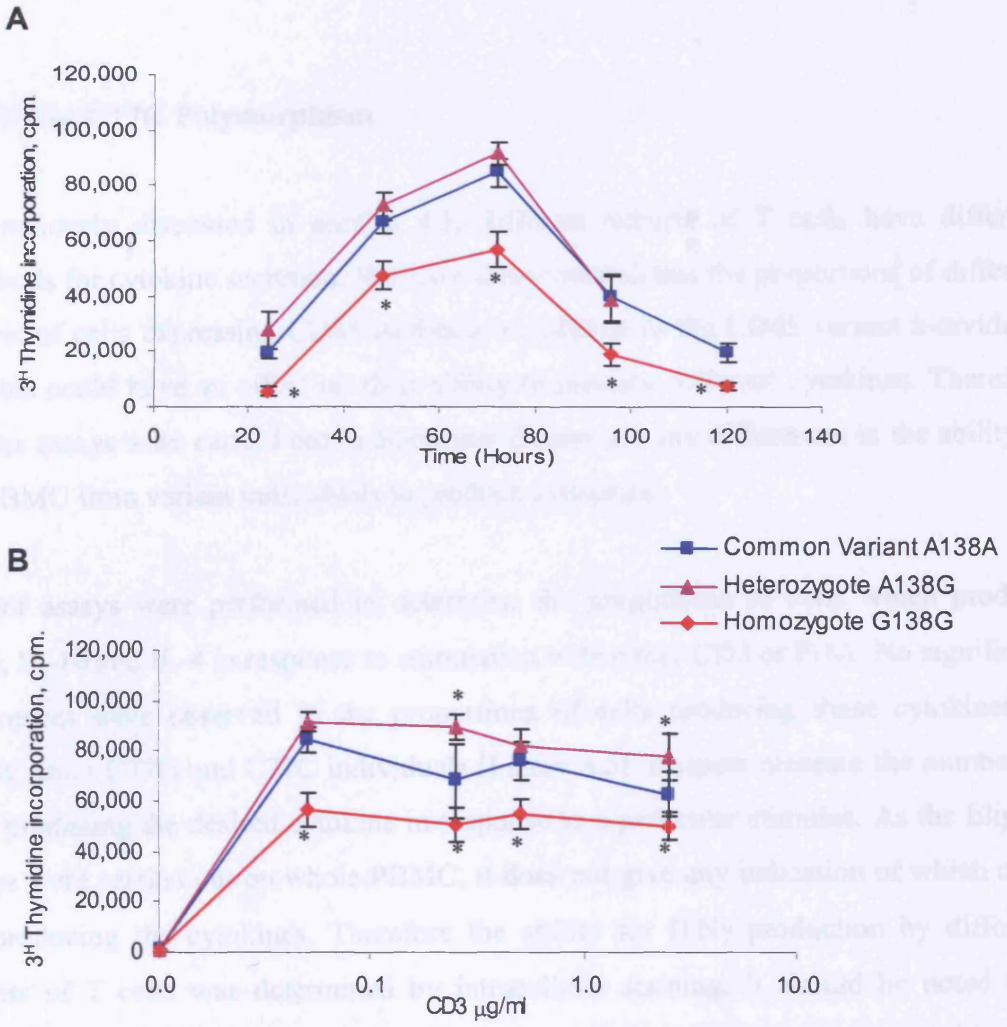



**Figure 4.4 Proliferative responses of PBMC from 4 G138G homozygous, 4 A138G heterozygous and 4 A138A common variant individuals.** Responses were assayed by thymidine incorporation. Results given are in counts per minute (cpm) with each sample being assayed in triplicate. Each line on the graph represents the mean of 4 individuals, with bars giving the standard error. (A) Response to 0.05µg/ml of anti-CD3 (UCHT1-2a) at varying time points. Background less than 2000cpm (data not shown). Analysis of variance (ANOVA) showed significant differences between the variant groups ($p=0.001$), and that the variance was different at each time point ($p=0.019$). Further analysis for each time point was carried out using Burnnett's test for multiple comparison; * $p<0.05$. (B) Response to varying doses of anti-CD3 (UCHT1-2a) at 72 hours. Analysis of variance (ANOVA) showed significant differences between the variant groups ($p=0.001$), and that the variance was dose dependant ($p=0.029$). Further analysis was carried out using Burnnett's test for multiple comparison, comparing each variant group to the control group at every dose; * $p<0.05$.

### 4.3.2 Cytokine production

#### 4.3.2.1 The C77G Polymorphism

As previously discussed in section 4.1, different subsets of T cells have differing potentials for cytokine secretion. We have demonstrated that the proportions of different subsets of cells expressing CD45 isoforms are altered in the CD45 variant individuals and this could have an effect on their ability to produce different cytokines. Therefore various assays were carried out to determine if there are any differences in the ability of the PBMC from variant individuals to produce cytokines.

Elispot assays were performed to determine the proportions of cells which produce IFNγ, IL-10 and IL-4 in response to stimulation with either CD3 or PHA. No significant differences were observed in the proportions of cells producing these cytokines in PBMC from C77G and C77C individuals (Figure 4.5). Elispots measure the number of cells producing the desired cytokine in response to a particular stimulus. As the Elipsot assays were carried out on whole PBMC, it does not give any indication of which cells are producing the cytokines. Therefore the ability for IFNγ production by different subsets of T cells was determined by intracellular staining. It should be noted that intracellular staining measures the potential of a cell to produce cytokines, and this may not entirely correlate with the amount of cytokine that is actually released from the cell.

Figures 4.6a and 4.6b show that, in accordance with the Elispot data, there is no significant difference in the IFNγ production by CD4 T cells between C77G and control individuals. The C77G CD8 cells, particularly the CD8+ CD45R0 + subset produce less IFNγ, but this difference is not significant (Figure 4.6d). These results show there is no significant difference in the proportion of PBMC secreting cytokines (IFNγ, IL-10, IL-4) or in the proportions of CD4 and CD8 T cells which have the capacity to produce IFNγ. However, the total number of cells producing cytokines is not necessarily a reflection of the quantity of cytokine being secreted. The cytokine content of the supernatants was determined using the human Th1/2 Cytokine Bead Array (CBA) kit (BD Pharmingen) for IFNγ, TNFα, IL-10, IL-5, IL-4 and IL-2. PBMC from CD45

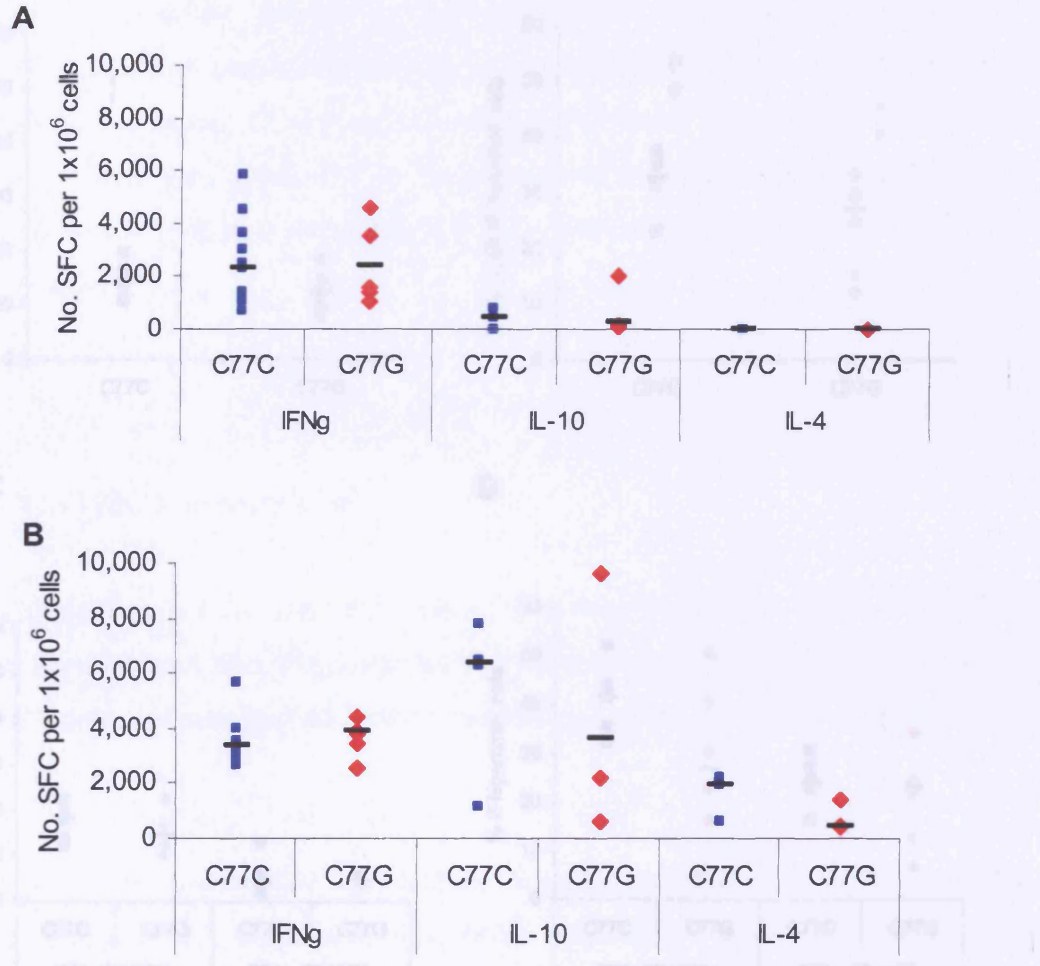



**Figure 4.5 Cytokine production by PBMC from heterozygous C77G and common variant C77C individuals.** Elispot assays were carried out to determine IFNγ, IL-10 and IL-4 production. Results are given as the number of spot forming cells (SFC) per $10^6$ cells. Each point on the graph represents the mean of triplicate samples from one individual, with between 4 and 10 individuals assayed for each cytokine. The horizontal bars show the median value for each variant or control group. (A) Response to 5 μg/ml CD3 (UCHT1-2a) at 48 hours. (B) Response to 10μg/ml of PHA-P at 48 hours.

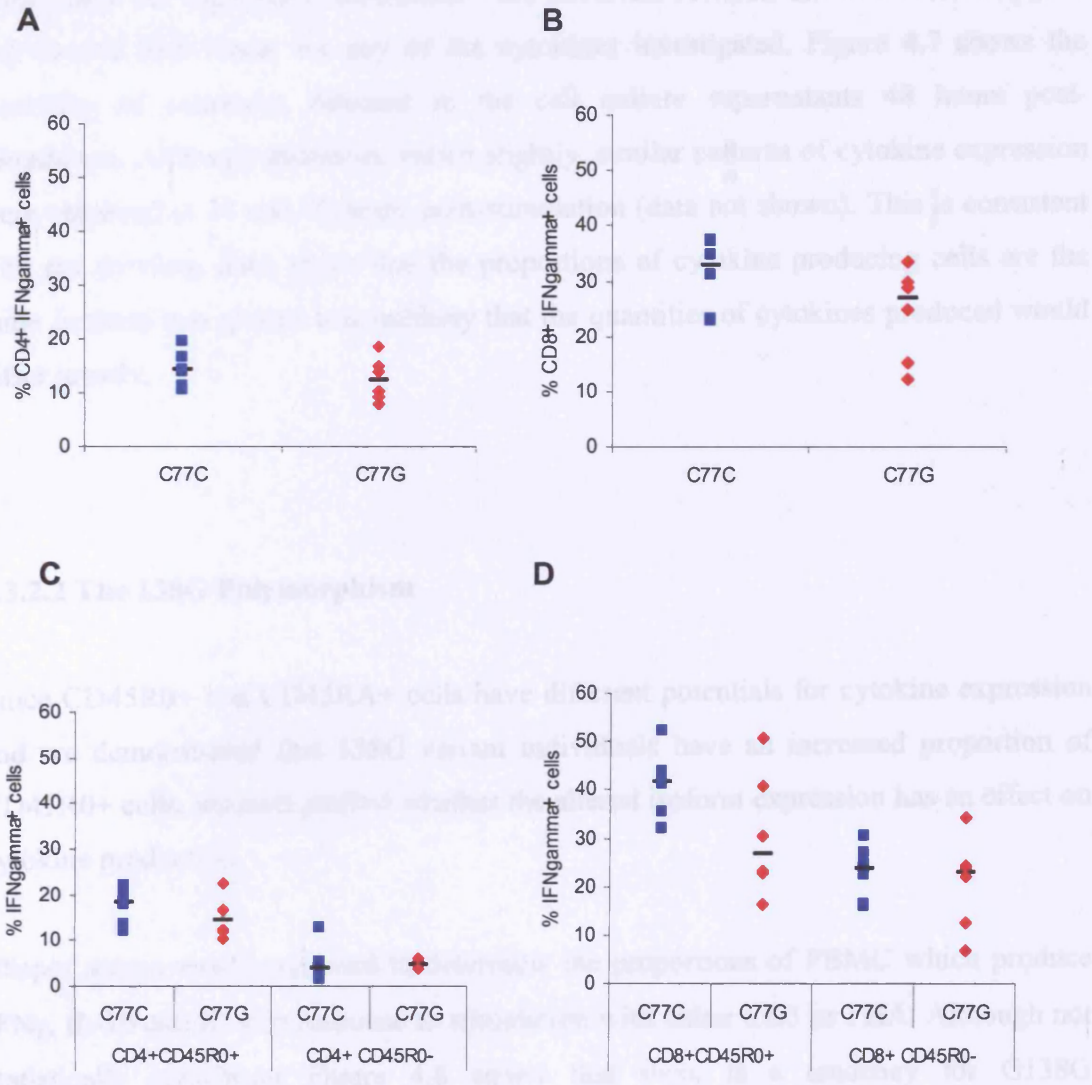



**Figure 4.6 Intracellular staining for IFNγ production.** PBMC were stimulated with 50ng/ml PMA and 500 ng/ml Ionomycin for 12 hours in the presence of Golgiplug (BD Pharmingen). Each point on the graph represents one individual and the horizontal bars show the median value for each group. IFNγ production in (A) CD4 cells and (B) CD8 cells from 6 C77G variant and 6 C77C control individuals. IFNγ production in (C) CD4+CD45R0+ and CD4+ CD45R0- cells and (D) CD8+CD45R0+ and CD8+ CD45R0- cells from 6 C77G variant and 6 C77C control individuals.

variant individuals and controls were stimulated with CD3 (UCHT1) or PMA and Ionomycin and cell culture supernatants were removed at 24, 48 and 72 hours post-stimulation. No significant differences were observed between the C77G heterozygous and control individuals for any of the cytokines investigated. Figure 4.7 shows the quantities of cytokines detected in the cell culture supernatants 48 hours post-stimulation. Although quantities varied slightly, similar patterns of cytokine expression were observed at 24 and 72 hours post-stimulation (data not shown). This is consistent with the previous data, given that the proportions of cytokine producing cells are the same in these two groups it is unlikely that the quantities of cytokines produced would differ greatly.

#### 4.3.2.2 The 138G Polymorphism

Since CD45R0+ and CD45RA+ cells have different potentials for cytokine expression and we demonstrated that 138G variant individuals have an increased proportion of CD45R0+ cells, we next studied whether the altered isoform expression has an effect on cytokine production.

Elispot assays were performed to determine the proportions of PBMC which produce IFNγ, IL-10 and IL-4 in response to stimulation with either CD3 or PHA. Although not statistically significant Figure 4.8 shows that there is a tendency for G138G homozygous individuals, with both CD3 and PHA stimulation, to have a slightly higher proportion of cells producing IFNγ. No difference was detected in the production of IL-10 or IL-4 between these two groups, at least not in the presence of a mainly Th1 stimuli.

To further analyse the differences in IFNγ production between the PBMC from 138G and 138A individuals, IFNγ production by different subsets of T cells was determined by intracellular staining. There is a significant difference in the percentage of both CD4 and CD8 cells producing IFNγ in G138G homozygous individuals, compared to A138A controls (Figure 4.9, $p<0.05$). As the phenotype of CD8 cells in CD45 variant

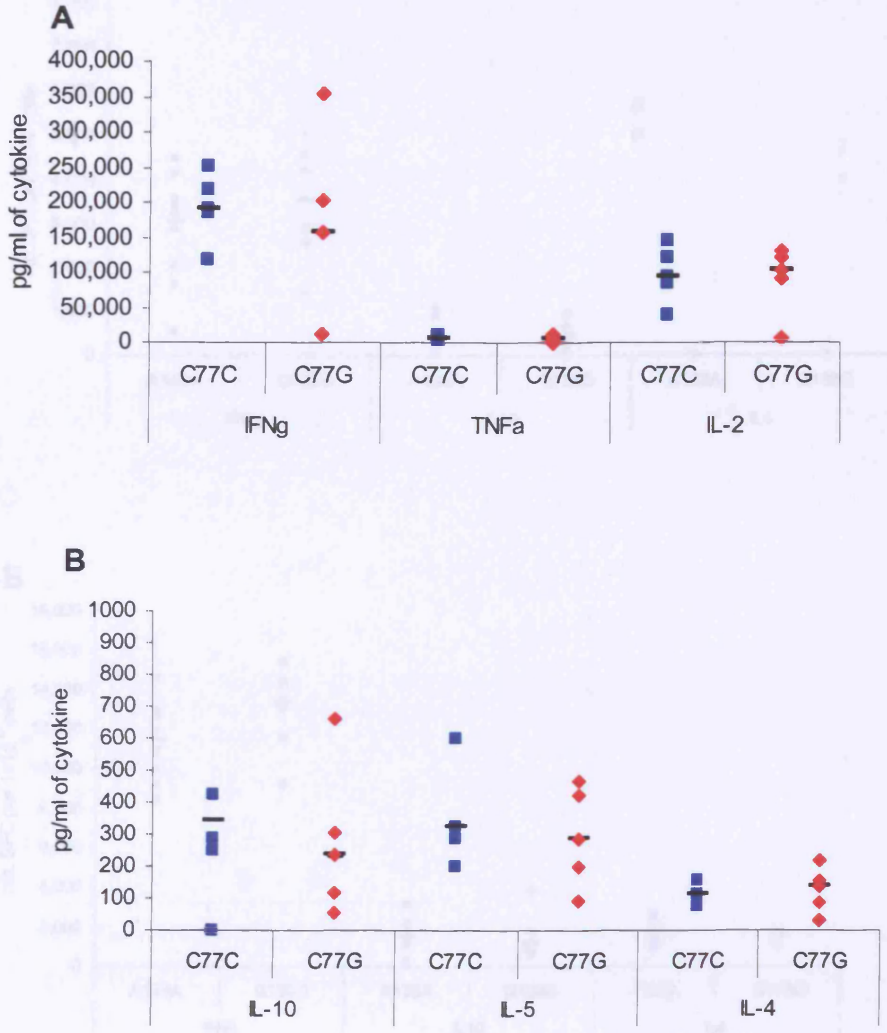



**Figure 4.7 Cytokine production by PBMC from 5 C77G heterozygous and 5 C77C common variant individuals as determined using the human Th1/2 Cytokine Bead Array (CBA) kit (BD Pharmingen).** PBMC were stimulated with 50ng/ml PMA and 500 ng/ml Ionomycin, and supernatants removed at 24, 48 and 72 hours. Each point on the graph represents the mean of triplicate samples from one individual. The horizontal bars show the median value for each variant or control group. Amount of (A) IFNγ, TNFα and IL-2 and (B) IL-10, IL-5 and IL-4 present in cell culture supernatants after 48 hours stimulation with PMA and Ionomycin. Similar trends were observed at 24 and 72 hours (data not shown).

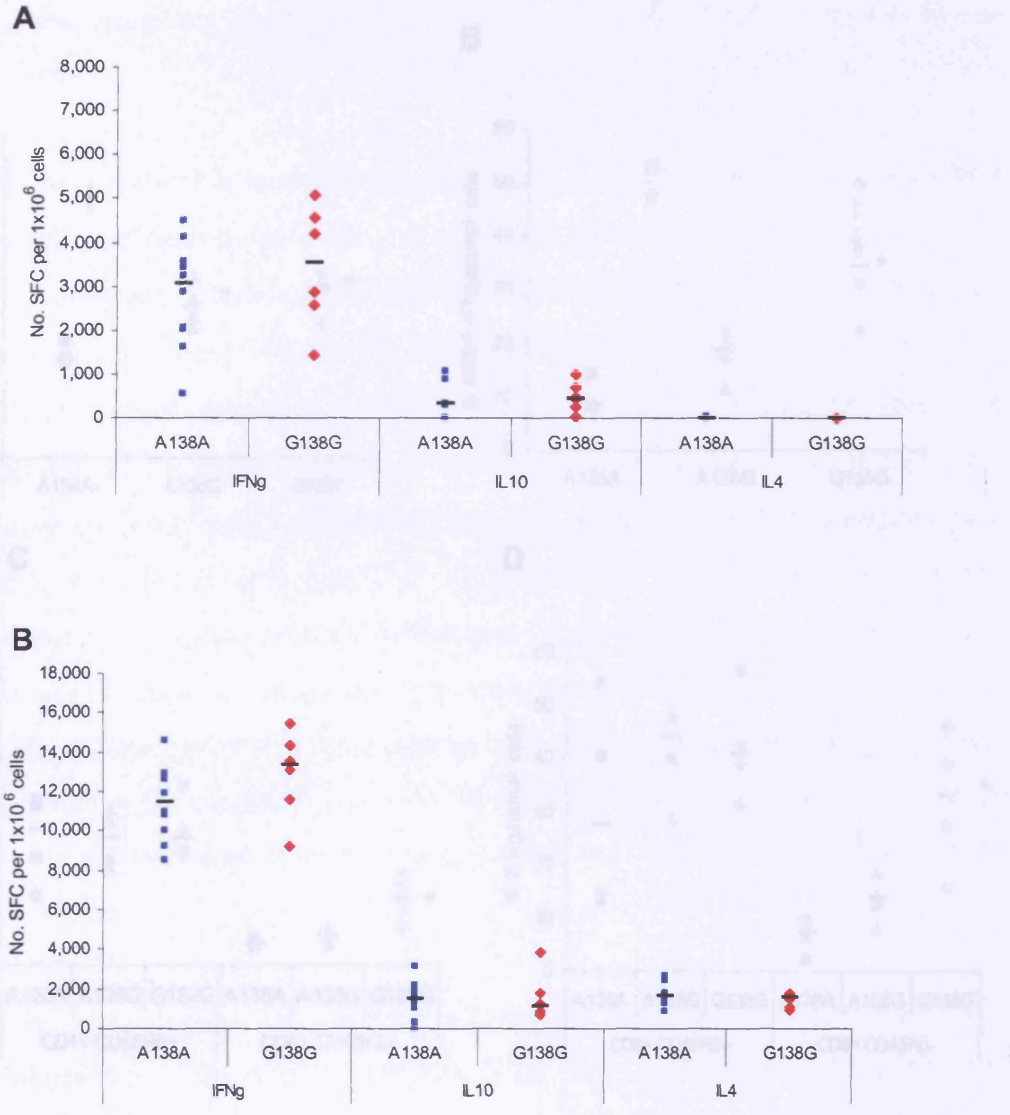



**Figure 4.8 Cytokine production by PBMC from G138G homozygous and A138A common variant individuals.** Elispot assays were carried out to determine IFNγ, IL-10 and IL-4 production. Results are given as the number of spot forming cells (SFC) per $10^6$ cells. Each point on the graph represents the mean of triplicate samples from one individual, with between 6 and 9 individuals assayed for each cytokine. The horizontal bars show the median value for each variant or control group. (A) Response to 5 μg/ml CD3 (UCHT1-2a) at 48 hours. (B) Response to 10μg/ml of PHA-P at 48 hours.

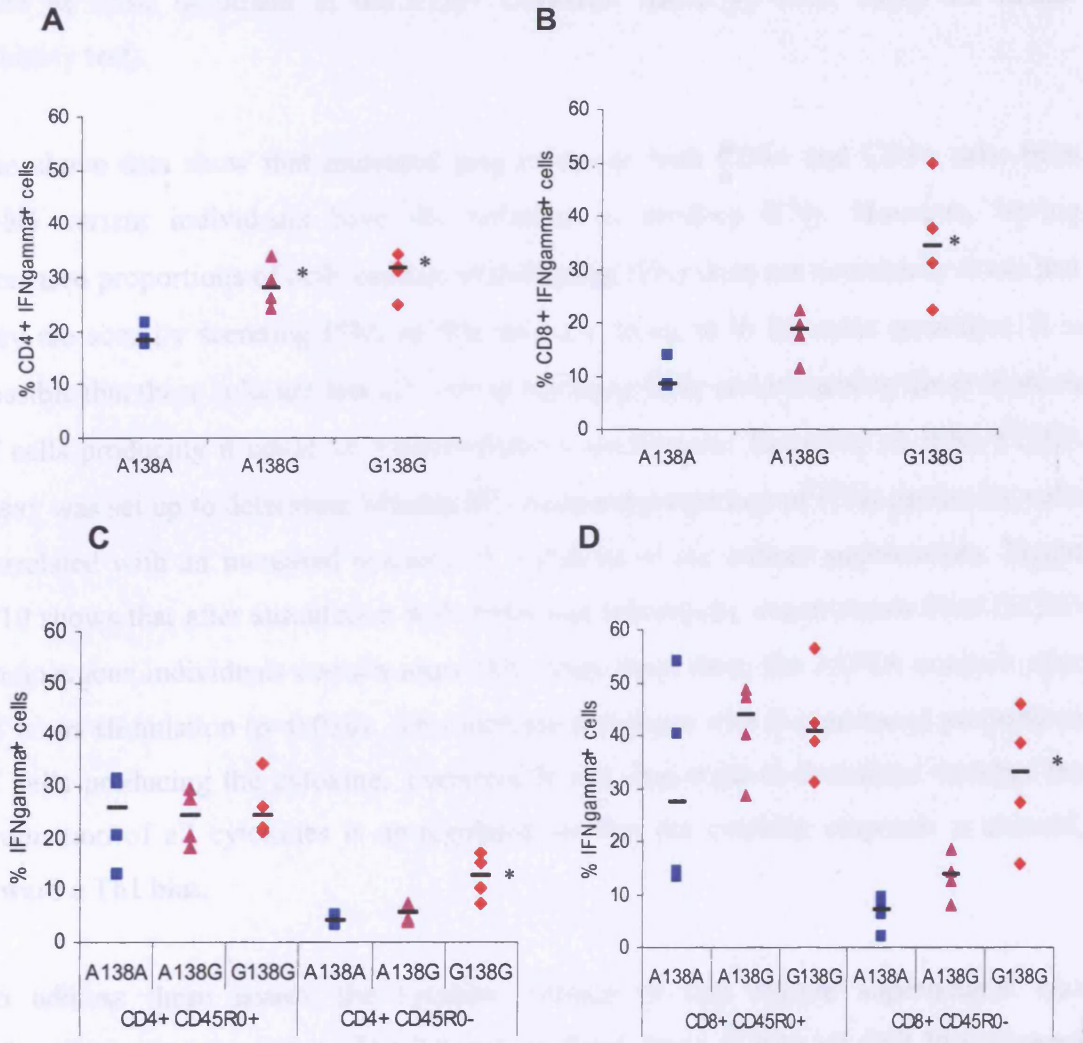



**Figure 4.9 Intracellular staining for IFNγ production.** PBMC were stimulated with 50ng/ml PMA and 500 ng/ml Ionomycin for 12 hours in the presence of Golgiplug (BD Pharmingen). Each point on the graph represents one individual and the horizontal bars show the median value for each group. Differences between G138G or A138G individuals and A138A controls were analysed using the Mann-Whitney test: * p=0.030. IFNγ production in (A) CD4 and (B) CD8 cells from A138G variant and A138A control individuals. IFNγ production in (C) CD4+CD45R0+ and CD4+ CD45R0- cells and (D) CD8+CD45R0+ and CD8+ CD45R0- cells from A138G variant and A138A control individuals. Differences between A138G or G138G and A138A individuals was analysed using the Mann-Whitney test:* p = 0.030.

individuals is always found to be more affected than the CD4 cells, it is not a surprise that the effects on IFNγ production are more pronounced in the CD8 cells. The effects were be more dominant in the CD8+ CD45R0- subset ($p<0.05$, using the Mann-Whitney test).

The above data show that increased proportions of both CD4+ and CD8+ cells from 138G variant individuals have the potential to produce IFNγ. However, having increased proportions of cells capable of producing IFNγ does not necessarily mean that they are actually secreting IFNγ or that they are doing so in the same quantities. It is possible that these cells are less efficient at releasing IFNγ and increasing the proportion of cells producing it could be a compensatory mechanism. Therefore an IFNγ ELISA assay was set up to determine whether the increased proportion of IFNγ producing cells correlated with an increased quantity of cytokine in the culture supernatants. Figure 4.10 shows that after stimulation with PMA and Ionomycin, supernatants from G138G homozygous individuals contain more IFNγ than those from the A138A controls after 48 hours stimulation ($p=0.030$). This increase correlates with the increased proportions of cells producing the cytokine. Therefore it was important to determine whether the production of all cytokines is up-regulated, or that the cytokine response is skewed, toward a Th1 bias.

To address these issues, the cytokine content of cell culture supernatants was determined using the human Th1/2 Cytokine Bead Array (CBA) kit (BD Pharmingen) to look at IFNγ, TNFα, IL-10, IL-5, IL-4 and IL-2. PBMC from CD45 variant individuals and controls were stimulated (with CD3 (UCHT1) or PMA and Ionomycin) and cell culture supernatants were removed at 24, 48 and 72 hours post-stimulation. Although there are variations in the quantity of cytokine produced, the pattern of the produced cytokines was similar at 24, 48 and 72 hours and with CD3 stimulation (data not shown). Figure 4.11 shows the quantity of IFNγ in the cell culture supernatants 48 hours post-stimulation with PMA and Ionomycin. Although not statistically significant, there is still a consistent trend for an increased amount of IFNγ produced after stimulation with PMA and Ionomycin, in G138G homozygous individuals. None of the other cytokines measured appeared to be different between the PBMC from G138G

homozygote and A138G common variant individuals, suggesting a particular effect on IFNγ production.

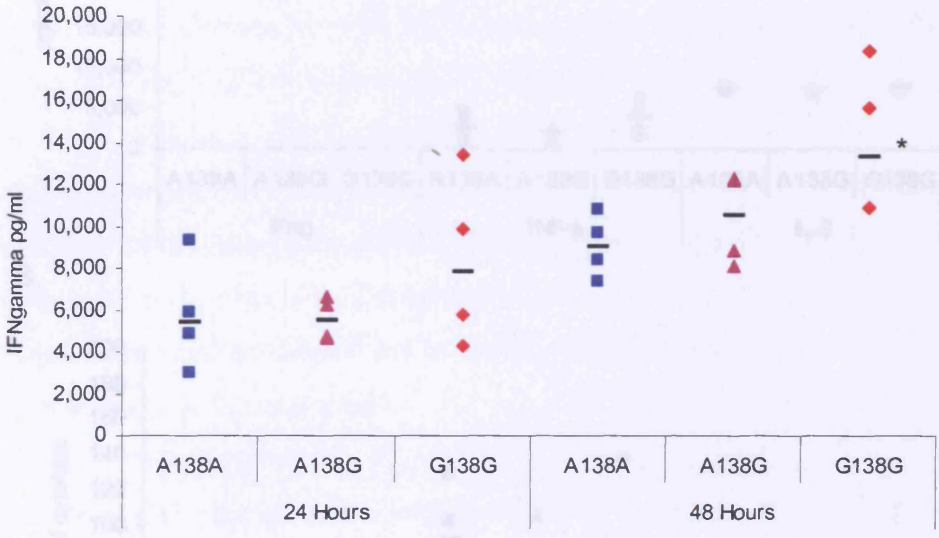



**Figure 4.10 IFNγ production by PBMC from 4 G138G homozygous, 4 A138G heterozygous and 4 A138A common variant individuals determined by Eliza.** PBMC were stimulated with 50ng/ml PMA and 500 ng/ml Ionomycin, and supernatants removed at 24 and 48 hours. Each point on the graph represents the mean of triplicate samples from one individual. The horizontal bars show the median value for each variant or control group. Differences between A138G or G138G and A138A individuals was analysed using the Mann-Whitney test:* $p = 0.030$.

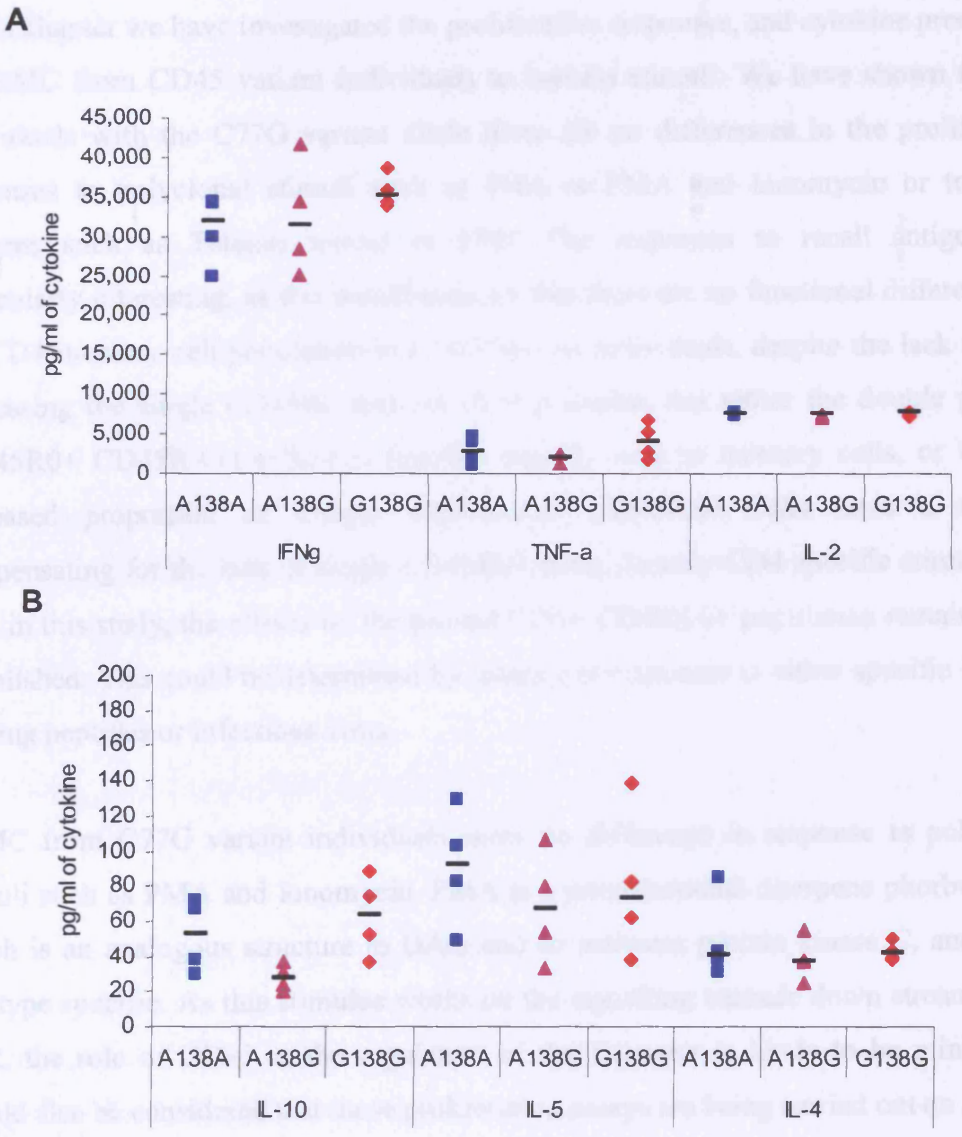



**Figure 4.11 Cytokine production by PBMC from 4 G138G homozygous, 4 A138G heterozygous and 4 A138A common variant individuals as determined using the human Th1/2 Cytokine Bead Array (CBA) kit (BD Pharmingen).** PBMC were stimulated with 50ng/ml PMA and 500 ng/ml Ionomycin, and supernatants removed at 24, 48 and 72 hours. Each point on the graph represents the mean of triplicate samples from one individual. The horizontal bars show the median value for each variant or control group. Amount of (A) IFNγ, TNFα and IL-2 and (B) IL-10, IL-5 and IL-4 present in cell culture supernatants after 48 hours stimulation with PMA and Ionomycin. Similar trends were observed at 24 and 72 hours (data not shown).

## 4.4 Discussion

In this chapter we have investigated the proliferative responses, and cytokine production of PBMC from CD45 variant individuals to various stimuli. We have shown that for individuals with the C77G variant allele there are no differences in the proliferative responses to polyclonal stimuli such as PHA or PMA and Ionomycin or to recall antigens such as Tetanus toxoid or PPD. The responses to recall antigens are particularly interesting, as this would indicate that there are no functional differences in the CD4 memory cell population in C77G variant individuals, despite the lack of cells expressing the single CD45R0 isoform. It is plausible that either the double positive (CD45R0+ CD45RA+) cells can function equally well as memory cells, or that the increased proportion of antigen experienced CD45RA+ cells have a role in compensating for the lack of single CD45R0+ cells. As only CD4 specific stimuli been used in this study, the effects on the primed CD8+ CD45RA+ population remains to be established. This could be determined by looking at responses to either specific MHC-1 binding peptides or infectious virus.

PBMC from C77G variant individuals show no difference in response to polyclonal stimuli such as PMA and Ionomycin. PMA is a polyfunctional diterpene phorbol ester, which is an analogous structure to DAG and so activates protein kinase C, and is not cell type specific. As this stimulus works on the signalling cascade down stream of the TCR, the role of CD45 in the regulation of the response is likely to be minimal. It should also be considered that these proliferation assays are being carried out on PBMC, so any effects on a single subset of cells, either in magnitude or kinetics of response, may be masked by the response of other cell types. It is therefore possible that either more T cell specific stimuli are needed or that separated subsets of cells should be used.

We have investigated the proliferative responses to CD3 stimulation in CD45 variant individuals. PBMC from C77G variant individuals show reduced proliferative responses to anti-CD3 stimulation ($p=0.04$). The fact that co-stimulation with CD3 and CD28 appears to restore the proliferative responses completely in C77G variant cells would suggest that these cells do have the same capacity to respond, but are possibly less efficient in doing so, perhaps due to an alteration in signalling threshold. This would be

further supported by the fact that higher doses of CD28 were required to restore the level of proliferative responses in PBMC from C77G variant individuals.

Given that primed CD8+ CD45RA+ cells have been shown to have a poor response to CD3 stimulation (Wills *et al.*, 1999), and there is a significant increase in the proportion of the CD45RA+ cells in the C77G variant individuals, it would possibly be expected that the responses in these individuals would be reduced. Perhaps the increased proportion of primed or antigen experienced CD45RA+ cells in these individuals can help to compensate for the lack of more responsive CD45R0+ cells, although further analysis of individual subsets would be required to really establish this.

Given that CD45R0+ T cells have been shown to be more responsive to CD3 stimulation (Robinson *et al.*, 1993), it is surprising that this was not observed in the proliferative response of PBMC from G138G homozygous individuals despite the increased proportion of CD45R0+ cells. It is possible that any effect of the increased proportion of CD45R0+ T cells is being masked by the presence of other cell types in the culture. It is also plausible that these extra CD45R0+ cells are more recently activated, and may not be in the same state of differentiation, or are more prone to apoptosis. However, stimulation of these cells with PMA and Ionomycin appears to restore the proliferative response to the same level as the A138A controls samples (data not shown) this would indicate that the PBMC from G138G variant individuals do have the same potential to respond. This suggests that these cells are defective or at least less efficient in signalling through the TCR. Although given the preliminary nature of this data, further analysis would be required to confirm this observation and determine the true cause of the reduced response.

One major factor for consideration when interpreting all of the proliferation data is that all of the samples used in these experiments have been cryopreserved PBMC. Comparisons of fresh and cryopreseved cells from healthy non-variant CD45 donors have shown that whilst the proportion of different cell types is preserved with cryopreservation, the magnitude of the proliferative response is not. We have shown that whilst the hierarchy of individual responses is preserved, i.e. the individual with the highest response to stimulation of fresh PBMC still gave the highest response after cryopreservation, the magnitude of the response from the cryopreserved PBMC was

significantly lower than that of the fresh samples (Figure 4.12). As all samples being compared in this study are cryopreserved this should not be a particular issue. However, it is not known if the effects of cryopreservation are the same in the CD45 variant cells, or even if a particular subset of cells is being selectively affected by the freezing process. It is possible that as the magnitude of the response is reduced the observed differences between the two groups are also reduced, making it harder to detect these at a significant level. Another consideration is the effect of cryopreservation on the cytokine production abilities of PMBC.

The data on cytokine production in cryopreserved PBMC is variable. In a series of studies on the effects of cryopreservation of cytokine secretion, Venkataraman showed that cryopreservation of PBMC induced enhanced production of IL-2 (1992), IL-6 (1994), IFNγ (1995), IL-10 (1996) and TNFα (1997) and decreased production of IL-12 (1996). These studies further suggested that the increase in IL-6 and IL-10 are an indirect result of the increase in TNFα production and that the observed decrease in IL-12 production is a direct result of the increased IL-10 production. This highly complex regulatory system may be a reason for the variability in the results observed from different studies. In contrast, Wang *et al.,* (1998) demonstrated that cryopreservation did not have an effect on the ability of PBMC to produce IFNγ, GM-CSF, TNFa or IL-6 in response to PHA stimulation. A further study by Kreher *et al.*, (2003) shows that cryopreservation of human PBMC does not have an effect on the production of IFNγ, IL-2, IL-4 or IL-5 in antigen specific T cells responses as determined by Elispot assays. Further to this we have shown by intracellular staining of PBMC from non-variant CD45 donors that there is no difference in the percentage of IFNγ producing cells after cryopreservation (data not shown).

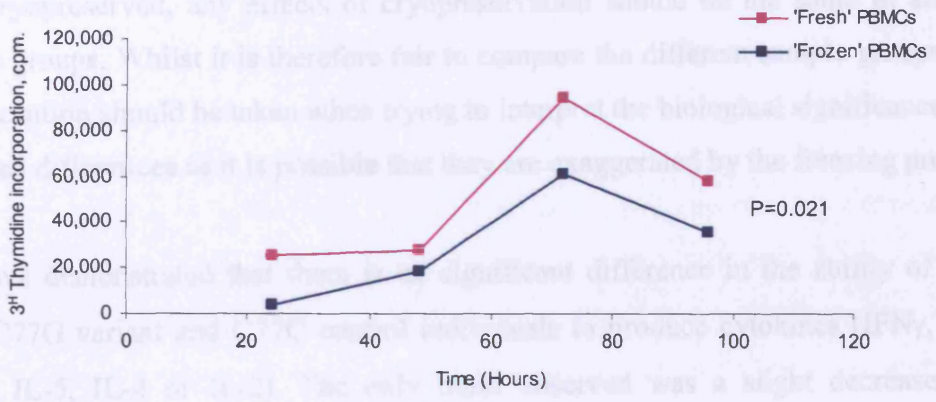



**Figure 4.12 Proliferative responses of PBMC from the same individual to 0.25mg/ml anti-CD3 (UCHT1-2a) before and after cryopreservation.** Responses were assayed by thymidine incorporation. Results given are in counts per minute (cpm) with each sample being assayed in triplicate. Background was less than 500 cpm (Data not shown). Figure representative of results from 4 healthy CD45 common variant individuals. Analysis of variance (ANOVA) was performed to determine the significance of the observed differences between the sample treatments (p=0.021).

However, the study of Wang *et al.*, (1998) did indicate a possible increase in IL-2 production in cryopreserved cells, which they suggest is caused by the DMSO used in cryopreservation. This may be particularly relevant as IL-2 is important in driving proliferation of cells. This may mean that in CD45 variant individuals rather than the altered proportions of cell subsets having a direct effect, the subtle effects observed are due to alterations in cytokine production. As all of the samples used in this study have been cryopreserved, any effects of cryopreservation should be the same in all of the sample groups. Whilst it is therefore fair to compare the different sample groups in this study, caution should be taken when trying to interpret the biological significance of any observed differences as it is possible that they are exaggerated by the freezing process.

We have demonstrated that there is no significant difference in the ability of PBMC from C77G variant and C77C control individuals to produce cytokines (IFNγ, TNFα, IL-10, IL-5, IL-4 or IL-2). The only trend observed was a slight decrease in the proportion of cells producing and the amount of IFNγ produced, but this was not found to be significant on this small sample group. However, PBMC from G138G variant individuals do have an increased proportion of cells, particularly CD8 cells, producing IFNγ. We have also observed increased quantities of IFNγ in cell culture supernatants after stimulation of PBMC from 138G variant individuals with either CD3 or PHA. Although this increase was consistent for the four individuals tested in this study, this is a small sample group, and caution should be taken when generalising such data. Interestingly no differences were found in any of the other cytokines tested, suggesting that it is not simply an up regulation of all cytokines but a specific up-regulation of IFNγ in these variant individuals. It is also worth noting that there does not appear to be any difference in IL-2 production in PBMC from G138G homozygous individuals, suggesting that this is not a cause of the observed reduction in proliferative response. Taken together these results suggest that altered CD45 isoform expression can affect both the proliferative and cytokine responses of T lymphocytes.

# CHAPTER 5

## CD45 mRNA expression in CD45 variant individuals and analysis of splicing using minigenes

### 5.1 Introduction

Alternative splicing is an important mechanism for controlling the expression of genes which encode multiple protein isoforms, by utilising different RNA processing pathways. These products can be produced by the use of alternative splice donor or acceptor sites, the inclusion or exclusion of variant exons or failure to remove introns (Breitbart *et al.,* 1987). Alternative splicing is regulated by both cis and trans-acting factors and the differential protein expression can be dependant on the tissue type (Laski *et al.,* 1986), stage of development (Breitbart and Nadal-Ginard, 1987) or sex (Nagoshi *et al.,* 1988).

Alternative splicing of CD45 exons 4, 5 and 6 (also referred to as exons isoforms A, B and C) at the N-terminus of the extracellular domain, could generate at least 8 different isoforms. Only 5 of these have been detected at the protein level in humans (Streuli *et al.,* 1987). There is some evidence at the RNA level, that exons 7, 8 and 10 can be alternatively spliced in murine T cells and T cell lines (Chang *et al.,* 1991; Virts *et al.,* 1998) although, protein expression has yet to be established. The exact function of the different CD45 isoforms remains obscure. The variable exons are heavily O-glycosylated, and alternative splicing of this region is a conserved feature of CD45 in a variety of species (Okumura *et al.,* 1996). This would suggest that the length of the region and alterations in the glycosylation pattern may be important for the function of CD45 and its interaction with other molecules.

The expression of CD45 isoforms is highly regulated and dependent upon the activation and differentiation states of haematopoietic cells (Novak *et al.,* 1994). In humans, it has

been used to distinguish between different functional subsets of lymphocytes. B cells express the high molecular weight (220kD) isoform CD45RABC (also called B220), whilst immature CD4+CD8+ T cells express mainly the low molecular weight isoforms. Mature CD4+ and CD8+ thymocytes and peripheral T cells can express multiple isoforms (Akbar *et al.,* 1988; Mackay, 1990; Ratech *et al.,* 1997). Generally naive T cells express the highest molecular weight (CD45RABC) isoform, and switch to express the lower molecular weight (CD45R0) isoform upon activation.

Tissue specific splicing of different CD45 isoforms is thought to be controlled by negatively regulatory trans-acting factors (Rothstein *et al.,* 1991) which allow the exons to be spliced out. However it has been shown that cis-elements within exons are also required for tissue specific alternative splicing (Streuli and Saito, 1989). Additionally it has been demonstrated that CD45 alternative splicing can be regulated by the antagonistic effects of SR protein splicing factors (ten Dam *et al.,* 2000). The tissue specific and activation state dependant expression of CD45 isoforms necessitates a complex regulatory system, so it is likely to be the quantitative effect of a number of different mechanisms which ultimately determines expression.

The feasibility of using CD45 mingenes to examine alternative splicing in different cell lines has previously been established. Streuli and Saito (1989) used minigene constructs containing the variably sliced exons 4, 5 and 6 to show that the same primary transcript is alternatively spliced in B cells and thymocytes. To establish that the exon 4 C77G point mutation was the cause of the abnormal splicing pattern observed in variant individuals, Zilch *et al.*, (1998) introduced the point mutation into a minigene construct containing exons 2, 3, 4, 7 and 8. The presence of this single point mutation was enough to strongly diminish splicing towards CD45R0, suggesting that it is the cause of the variant splicing pattern.

Lynch and Weiss (2001) have described several splicing regulatory elements within exon 4, which increase (nucleotides 169-188) or decrease (nucleotides 89-108 and 149-168) the inclusion of exon 4 during splicing. The strongest of the regulatory elements was the exonic splice silencer (nucleotides 29-88) designated ESS1, which normally represses the inclusion of exon 4, by inhibiting the use of the weak 5' splice site. Lynch

and Weiss confirmed previous observations (Zilch *et al.,* 1998) that the introduction of the C77G point mutation into a minigene containing exons 3, 4 and 7, almost completely abolishes the splicing out of exon 4. They associated this abnormal splicing with the disruption of the strong exonic splice silencer, ESS1.

Tsai *et al.*, (1989) used a construct containing exons 2, 6 and 8 to investigate which portions of the exon 6 sequence are essential for tissue specific alternative splicing. Using a series of substitution mutations, they observed that altering the last 10bp of the exon 6 sequence, lead to skipping of the exon 6 pre-mRNA and therefore the exon was always spliced out both in T and B cell lines. Considering these results in the light of the observation that individuals with the 138G variant allele, show an increase in the proportion of lower molecular weight isoforms expressed, it is plausible that the point mutation may promote the splicing out of exon 6.

Whilst several sequence elements have been described which are important in the basal level of splicing in resting cells, the sequences responsible for mediating activation-induced exon repression are largely unexplored. Rothrock *et al.,* (2003) described a conserved signal-responsive sequence that mediates activation induced alternative splicing of CD45. This study found that the 60 nucleotide ESS1 sequence in exon 4 is sufficient to confer PMA-induced exon repression. Rothrock et al., (2003) showed that there is an imperfect repeat sequence which is conserved between ESS1, exon 5 and exon 6, and murine exon 7, called the activation-responsive sequence (ARS). It is unlikely that the ARS consensus sequence alone is sufficient to confer signal induced exon skipping in all contexts, although it is an important regularly element and has been found in other alternatively spliced exons, such as the transmembrane exon of CTLA-4. Interestingly this study also showed that the C77G point mutation does not have any effect on the ARS function of the ESS1 element. This means that whilst the C77G polymorphism reduces the basal splice silencing activity of ESS1 (Lynch and Weiss, 2001), it does not have an effect on ARS function, and therefore PMA-induced repression.

## 5.2 Objectives

This chapter has two objectives, the first is to analyse CD45 mRNA expression in PBMC from CD45 variant individuals and determine if the altered isoform expression was due to an effect of the variant allele on splicing. The second objective is to use minigene constructs to determine if the introduction of the exon 6 138G mutation alters splicing, and thus CD45 isoform expression.

## 5.3 Results

### 5.3.1 CD45 mRNA expression in C77G variant individuals

Individuals with the C77G variant allele constitutively express CD45RA on their T cells even after mitogen stimulation. Immunoprecipitation assays and RT-PCR analysis of stimulated T cells from variant individuals determined that whilst splicing of the 220kD CD45RABC isoform was unaffected, the 205kD CD45RAB isoform lacks proper regulation (Schwinzer *et al.*, 1992). We performed RT-PCR analysis on unstimulated PBMC to determine the expression pattern of CD45 mRNA in individuals with the C77G polymorphism.

Total RNA was extracted from unstimulated PBMCs and after reverse transcription the cDNA was amplified with specific CD45 primers spanning exons 2 to 7. Interestingly a faint band corresponding to the CD45RA transcript (393bp) was observed in the C77G variant samples, which was undetectable in the controls (Figure 5.1). The presence of this band was observed in all 4 of the variant individuals tested.

The relative proportion of each transcript was determined by quantifying the size of each band as a percentage of the total bands in the sample. Figure 5.2 gives the mean values obtained from 4 C77G variant and 4 C77C common variant individuals. In agreement with previously published data (Schwinzer *et al.*, 1992) there is a significant difference in the proportion of expressed message for the CD45RAB isoform, with increased transcript expression for the CD45RAB (26.5 +/- 3.7) and corresponding decrease in expression of CD45RB (20.5 +/- 4.9) transcript in C77G variant individuals ($p=0.03$).

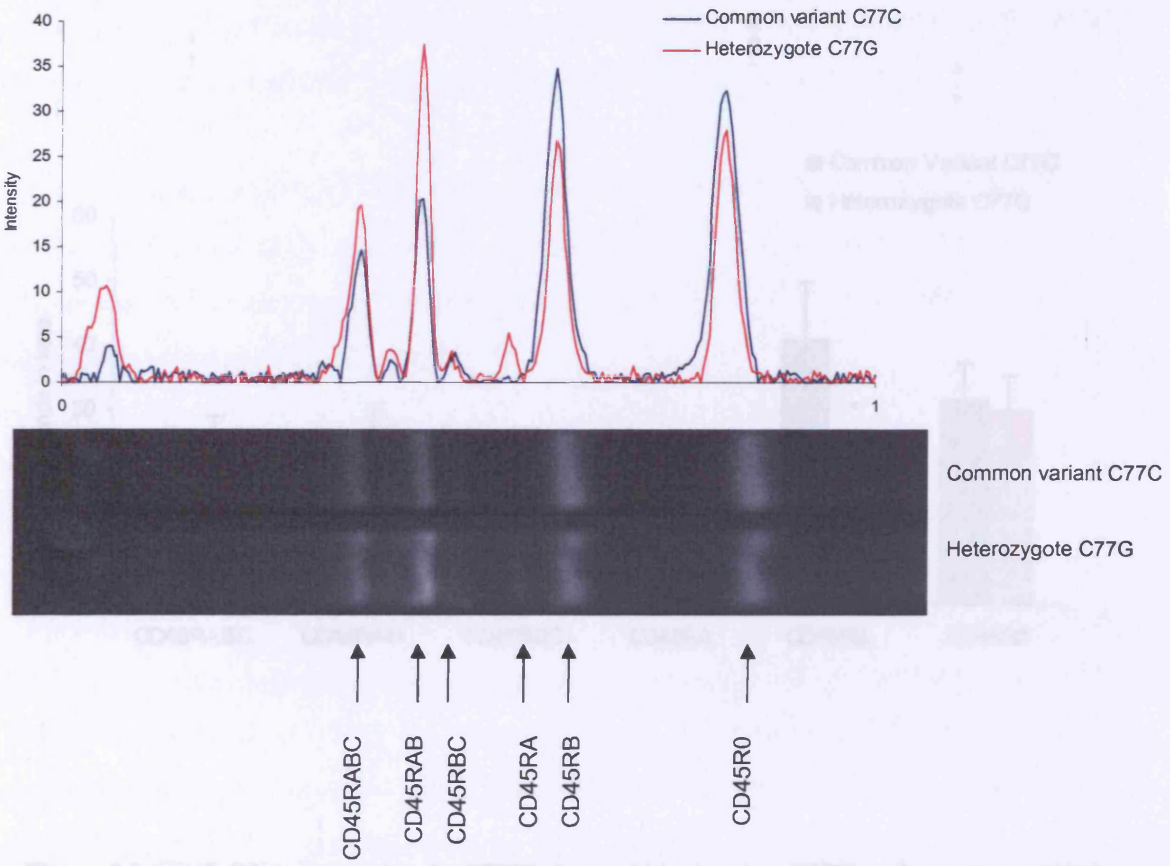



**Figure 5.1 CD45 cDNA expression in PBMC from C77G and C77C individuals.** Total RNA was extracted from unstimulated PBMC. After reverse transcription, the cDNA was amplified with primers spanning exons 2-7 of the CD45 gene. PBMC from individuals with the variant C77G or common C77C alleles contained mRNA for the CD45R0 (197bp), CD45RB (337bp), CD45RA (393bp), CD45RBC (480bp), CD45RAB (534bp), and CD45RABC (677bp) isoforms. The bands in each line were quantified and the corresponding densitograms shown above. Data shown is representative of duplicate analysis of 4 variant C77G and 4 control C77C individuals.

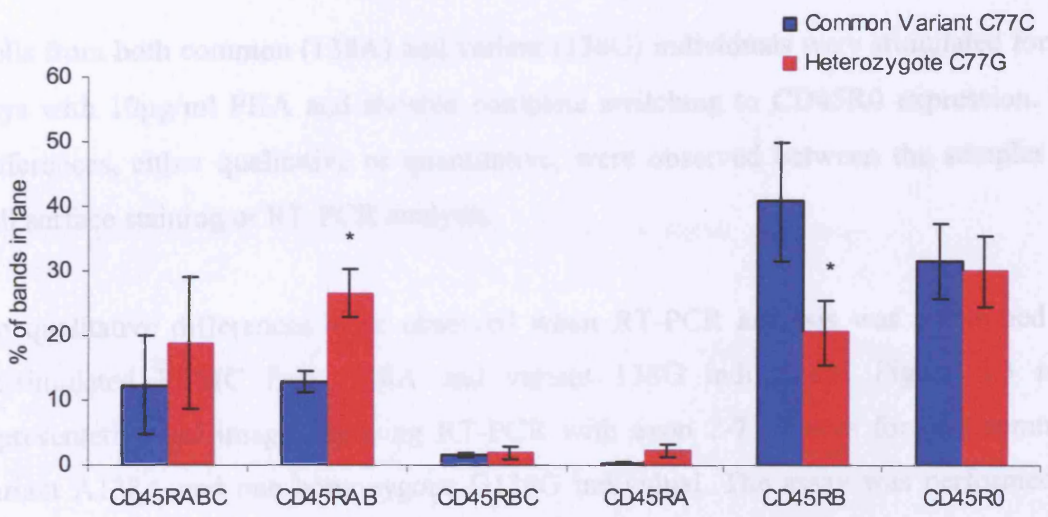



**Figure 5.2 CD45 RNA expression in PBMC from heterozygous C77G and common variant C77C individuals.** Total RNA was extracted from unstimulated PBMC's. After reverse transcription, the cDNA was amplified with primers spanning exons 2-7 of the CD45 gene. The bands in each line were quantified and graph above shows the expression of each isoform as a percentage of the bands present in each lane. The values are the mean and standard deviation of 4 samples ($p = 0.030$ using Mann Whitney Test).

### 5.3.2 CD45 mRNA expression in 138G variant individuals

As discussed previously in chapter 3, we have shown that individuals with the exon 6 138G variant have a higher proportion of T cells expressing CD45R0, in either the presence or absence of CD45RA. To establish whether the alteration in CD45R0 expression is a direct result of the variant 138G allele interfering with splicing, RT-PCR analysis was performed on PBMC before and after stimulation with PHA.

Cells from both common (138A) and variant (138G) individuals were stimulated for 10 days with 10μg/ml PHA and showed complete switching to CD45R0 expression. No differences, either qualitative or quantitative, were observed between the samples by cell surface staining or RT-PCR analysis.

No qualitative differences were observed when RT-PCR analysis was performed on unstimulated PBMC from 138A and variant 138G individuals. Figure 5.3 is a representative gel image, showing RT-PCR with exon 2-7 primers for one common variant A138A and one homozygous G138G individual. The assay was performed in duplicate on four individuals of each genotype. Although there were no qualitative differences in terms of CD45 isoforms expressed, on quantifying the intensity of the bands a difference in the level of the CD45R0 transcripts was found. This can be clearly seen in the corresponding densitogram of the gel image.

Quantifying the size of each band as a percentage of the total bands in the sample, gives an indication of the relative proportion of each transcript (Figure 5.4). In PBMC from individuals with the A138A common variant, the ratio of the CD45RB to CD45R0 transcripts is 0.93 (+/- 0.08) whilst for A138G heterozygous individuals it is 0.78 (+/- 0.33) and for G138G homozygous individuals it is 0.47 (+/- 0.34). These results suggest that the 138G variant has a quantitative effect on splicing, with these individuals expressing more CD45R0 transcript than the controls.

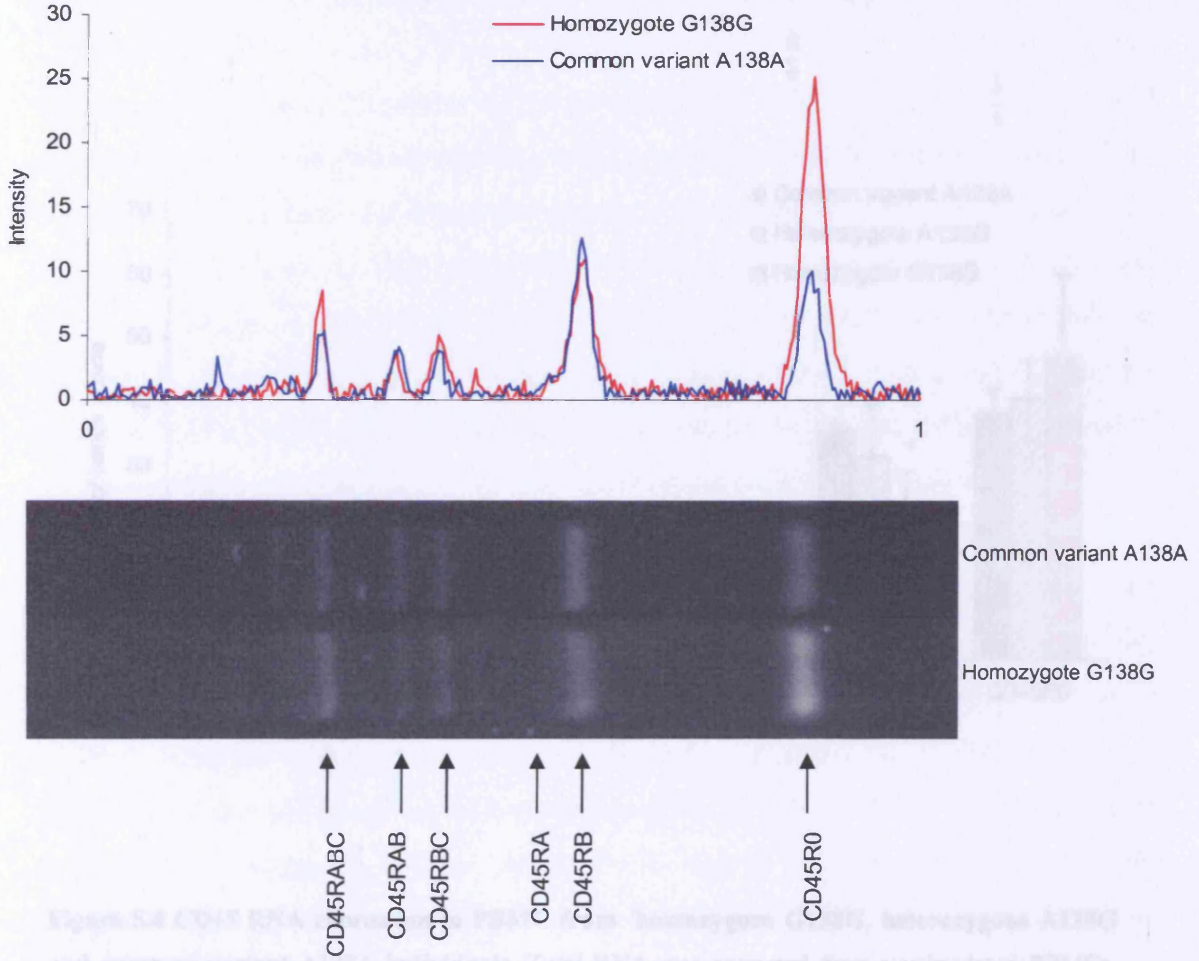



**Figure 5.3 CD45 RNA expression in PBMC from homozygous G138G and common variant A138A individuals.** Total RNA was extracted from unstimulated PBMC. After reverse transcription, the cDNA was amplified with primers spanning exons 2-7 of the CD45 gene. PBMC from individuals homozygous for the variant G138G or common A138A alleles contained mRNA for the CD45R0 (197bp), CD45RB (337bp), CD45RA (393bp), CD45RBC (480bp), CD45RAB (534bp), and CD45RABC (677bp) isoforms. The bands in each line were quantified and the corresponding densitograms shown above. Data shown is representative of duplicate analysis of 4 A138A and 4 G138G homozygous individuals.

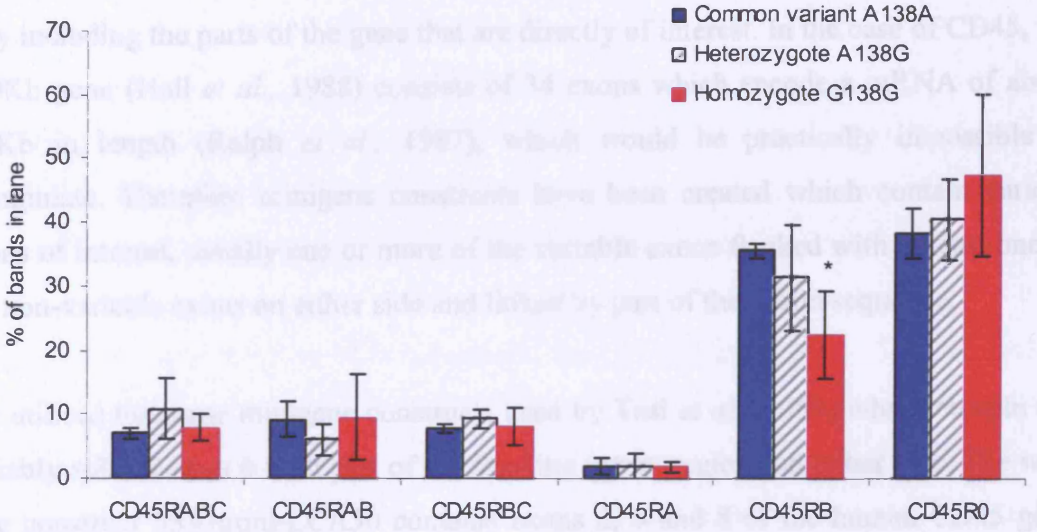



**Figure 5.4 CD45 RNA expression in PBMC from homozygous G138G, heterozygous A138G and common variant A138A individuals.** Total RNA was extracted from unstimulated PBMCs. After reverse transcription, the cDNA was amplified with primers spanning exons 2-7 of the CD45 gene. The bands in each line were quantified and the expression of each isoform is shown as a percentage of the bands present in each lane. The values are the mean of 4 samples, with error bars giving standard deviation. * p= 0.051 (comparison of G138G homozygous to A138A common variant individuals using Mann Whitney Test).

### 5.3.3 Minigene analysis of the exon 6 138G variant allele

Both the phenotypic data (chapter 3) and analysis of mRNA level suggested an increase in CD45R0 expression in 138G variant individuals. Therefore we wanted to establish whether the 138G variant allele is the cause of this variant splicing.

The use of minigene constructs to study alternative splicing mechanisms offers a distinct advantage, they allow normally large genes to be manipulated and analysed by only including the parts of the gene that are directly of interest. In the case of CD45, the 130Kb gene (Hall *et al.,* 1988) consists of 34 exons which encode a mRNA of about 5.2Kb in length (Ralph *et al.,* 1987), which would be practically impossible to manipulate. Therefore minigene constructs have been created which contain various exons of interest, usually one or more of the variable exons flanked with at least one of the non-variable exons on either side and linked by part of the intron sequence.

We utilised the same minigene constructs used by Tsai *et al,* (1989) which contain the variably spliced exon 6 with part of the flanking intron regions on either side. The wild type construct pSV-mini-LCA30 contains exons 2, 6 and 8 of the human CD45 gene under the control of the simian virus (sv) 40 early promoter. The LS37 construct is identical to the pSV-mini-LCA30 construct except that it has 10bp substitution mutations of nucleotides 134 to 144 at the 3' end of exon 6 (Figure 5.5a). Both constructs were kindly provided by Dr H. Saito. The A138G point mutation was introduced into the pSV-mini-LCA30 construct by site directed mutagenesis using a high fidelity Pfu-PCR based method, to create the LS138 construct.

The three constructs were transiently transfected by electroporation into COS-7 cells. After 48 hours the cells were harvested and total RNA extracted. After reverse transcription the cDNA was amplified using specific CD45 exon 2 and 8 primers and run out on a Visigel separation matrix (Figure 5.6). The two possible alternative splice products contain exons 2-6-8 (266bp) and exons 2-8 (122bp), as shown in figure 5.5b. Six separate transfections were carried out for each construct and Figures 5.7 shows one representative clone for each construct.

As previously demonstrated by Tsai *et al.*, the wild type pSV-mini-LCA30 construct was alternatively spliced in COS-7 cells to give two products, exon 2-6-8 and exon 2-8, with the larger exon 2-6-8 being predominant. The LS37 construct also gave the two splice products, but was extremely biased towards production of the smaller exon 2-8 splice product. The observed exon 2-6-8 band for the LS37 construct is slightly smaller then for the wild type pSV-mini-LCA30 construct, this is likely due to shortening during construction of the linker substitution mutants (Tsai *et al.,* 1989). Although attempts were made to confirm the sequence of this band, these were unsuccessful due to the small quantity of the PCR product.

The LS138 construct produced both splice products, and whilst it was not completely biased as with the LS37 construct, there was an increase in the proportion of the exon 2-8 splice product compared to the controls. The ratio of the exon 2-6-8 to exon 2-8 bands was calculated for each transfection and the mean of six calculated for each construct. The exon 2-6-8 to exon 2-8 ratio of the LS138 construct was 6.4 compared to 12.1 for the wild type construct and 0.6 for the LS37 construct. This indicates that although it does not cause the constitutive splicing out of exon 6, the 138G point mutation has a quantitative effect on the splicing pattern, with the ratio of the two splice products being almost half that observed with the wild type construct.

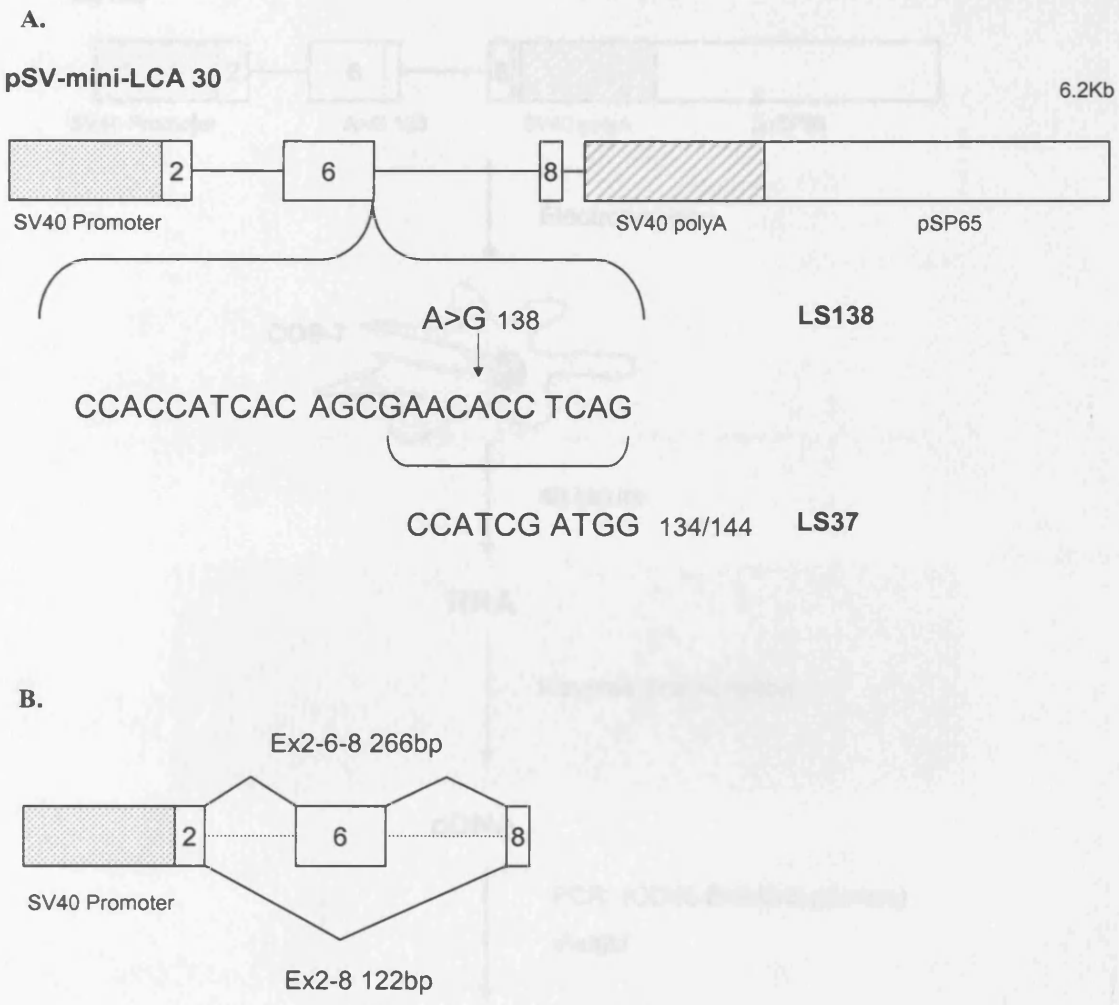



**Figure 5.5 The effect of the exon 6 138G mutation on splicing.** (A) Schematic representation of the minigene constructs used.The wild-type pSV-mini-LCA30 construct contains the variable exon 6 flanked by the constitutive exons 2 and 8. The exons are represented by open boxes, and the introns are represented by lines (not drawn to scale). The mutant LS37 has a 10bp substitution at nucleotides 134-144 at the 3' end of exon 6 (Tsai *et al.*, 1989). As indicated in the schematic, the mutant LS138 was created by introducing a point mutation (A to G) at position 138 of Exon 6. (B) The possible alternative splice products are indicated, with expected sizes.

**Transfection of COS-7 cells with CD45 minigenes**

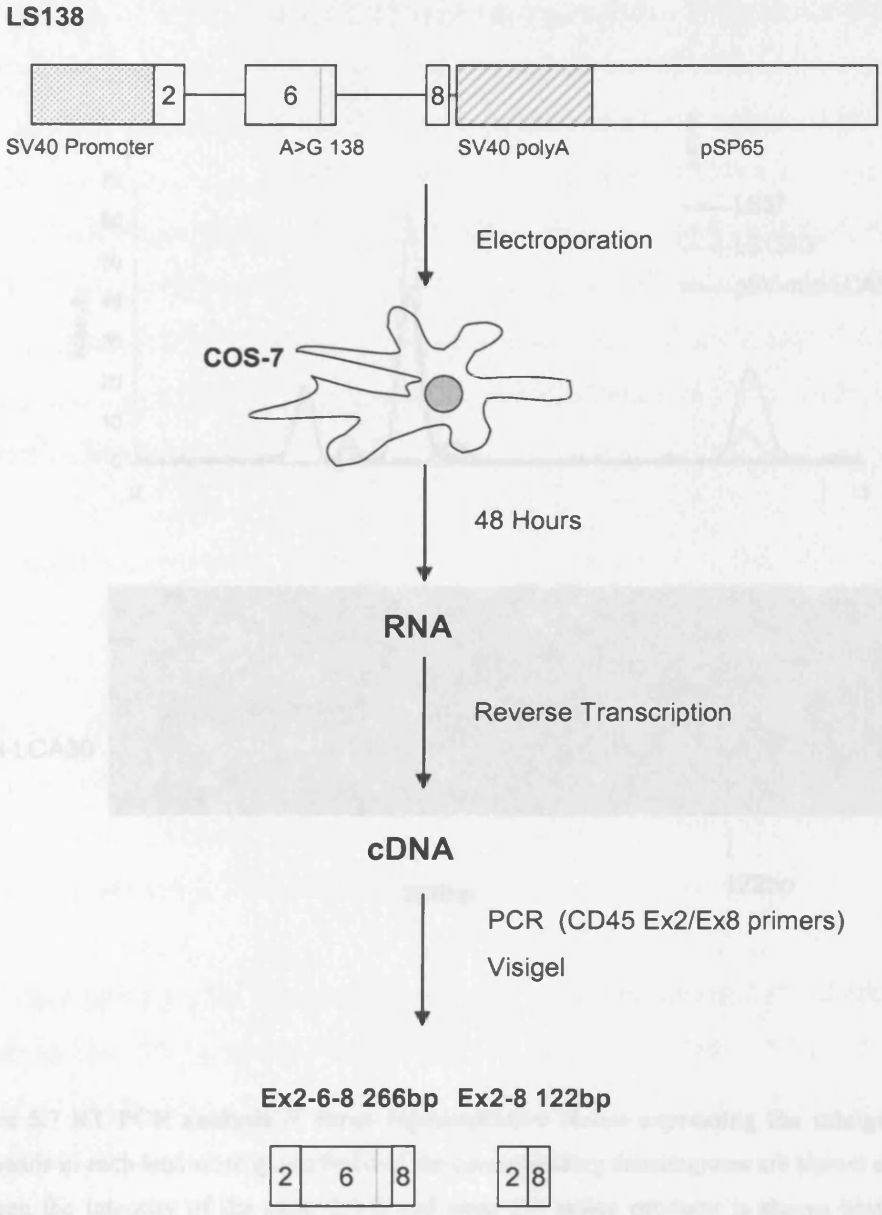



**Figure 5.6 Schematic representation of the CD45 minigene splicing assay.** The LS138 minigene construct is represented as boxes (exons, with number specification inside) and lines (introns). One microgram of the minigene construct was transiently transfected into COS-7 cells by electroporation. After 48hours, total RNA was extracted from the cells and reverse transcribed. The cDNA was amplified using CD45 Exon 2 and 8 specific primers. The amplified products were run out on a Visigel separation matrix and the bands quantified (See figure 5.7).

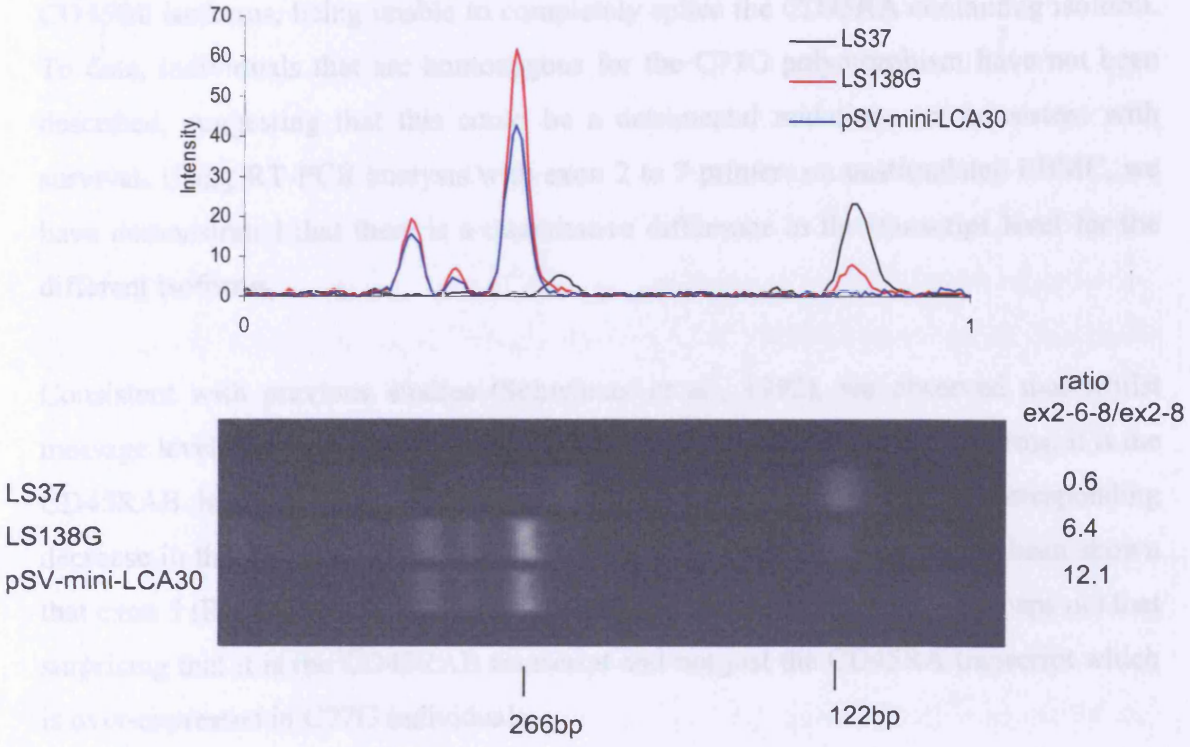



**Figure 5.7 RT-PCR analysis of three representative clones expressing the minigenes indicated.** The bands in each lane were quantified and the corresponding densitograms are shown above. The ratio between the intensity of the exon 2-6-8 and exon 2-8 splice products is shown beside the gel and represents the mean of six transfections for each minigene. The bands of the expected size (266bp for exon 2-6-8 and 122bp for exon 2-8) were cloned and sequenced to confirm that they were the correctly spliced products. Larger than expected bands were also detected, these are most likely unspliced RNA.

## 5.4 Discussion

In this chapter, the RNA expression of CD45 isoforms has been determined in PBMC from individuals with variant CD45 isoform expression. Individuals with the exon 4 C77G polymorphism show constitutive expression of CD45RA containing isoforms. Upon mitogen stimulation PBMC from these individuals co-express both CD45RA and CD45R0 isoforms, being unable to completely splice the CD45RA containing isoform. To date, individuals that are homozygous for the C77G polymorphism have not been described, suggesting that this could be a detrimental mutation, not consistent with survival. Using RT-PCR analysis with exon 2 to 7 primers on unstimulated PBMC, we have demonstrated that there is a quantitative difference in the transcript level for the different isoforms.

Consistent with previous studies (Schwinzer *et al.,* 1992), we observed that whilst message levels are increased for both the CD45RABC and CD45RAB isoforms, it is the CD45RAB isoform which is predominantly affected. There is also a corresponding decrease in the expression of CD45RB. Of the three variable exons, is has been shown that exon 5 (B) is the least tightly regulated (Streuli *et al.,* 1987), so it is perhaps not that surprising that it is the CD45RAB transcript and not just the CD45RA transcript which is over-expressed in C77G individuals.

When interpreting data from this study it is important to remember that we are describing isoform expression on PBMC, and as isoform expression is known to be cell type specific the proportions of different cell types will have an effect on the observed transcript. In chapter 3 we demonstrated that the proportions of different cell types were not altered in CD45 variant individuals. Therefore it is highly unlikely that any differences observed are influenced by an alteration in the proportion of cells, but it is possible that either the expression pattern or ratios of different isoforms may be altered when describing a specific cell type. This is particularly relevant when considering T cells, where other factors such as age and health status can have an influence on isoform expression.

We have shown both by flow cytometry and RT-PCR analysis, that PBMC from 138G variant individuals have increased expression of CD45R0. Changes in this region of

exon 6 have been shown to affect isoform expression, promoting the skipping of exon 6 during splicing (Tsai *et al.,* 1989). Therefore we would suggest that the A to G substitution in exon 6 of these variant individuals is the cause of the observed changes in isoform expression. To establish this, we utilised the minigene constructs used by Tsai *et al.,* which contain exons 2, 6 and 8. We introduced the A138G point mutation into the wild type pSV-mini-LCA30 construct to create the LS138 construct. These constructs, along with the previously described LS37 construct which contains the 10bp substitution in exon 6, were transiently transfected into COS-7 cells. RT-PCR analysis produced two alternatively spliced products. Consistent with the previous study, the wild type construct gave both splice products, with the larger exon 2-6-8 product being predominant. The LS37 construct almost exclusively spliced to the exon 2-8 product, showing the preferential exclusion of exon 6. Introducing the A138G point mutation did have a quantitative effect on splicing, with the LS138 construct showing an increase in the splicing out of exon 6 compared to the wild type control. This data provides direct evidence that it is the 138G mutation which is the cause of the observed alteration in splicing and therefore CD45R0 isoform expression in 138G variant individuals.

There are some disadvantages of using such an artificial system for studying splicing mechanisms. The first of these is that the exons used in the constructs are not always those which would be found adjacent to each other, and therefore they may not behave in exactly the same manner. This is also relevant as large portions of the intron sequences are omitted from the constructs, and it is possible that there are some regulatory elements found within the introns which have been removed.

The model proposed by Tsai *et al.*, (1989) suggests that the 10bp substation in exon 6 reduces the overall similarity of the splice site to the consensus sequence, which would result in less efficient recognition by the spliceosome. It is plausible that the A138G polymorphism would work in a similar manner, with a slight reduction on the similarity of the sequence causing a slight decrease in efficiency. However it is also possible that the A138G polymorphism may disrupt an as yet unknown regulatory sequence within the exon, as with the disruption of the ESS1 sequence by the C77G polymorphism. Clearly further analysis would be needed to properly define the complex splicing mechanisms involved.

# CHAPTER 6

## CD45 isoform expression in individuals with 'novel' CD45 polymorphisms

### 6.1 Introduction

The best characterised polymorphism in the PTPRC gene is the previously discussed C77G transversion in exon 4. This polymorphism disrupts a strong exonic splice silencer, preventing splicing of exon 4, leading to the expression of both high and low molecular weight CD45 isoforms on memory T cells. The C77G variant allele has been found at increased frequency in HIV-1 positive individuals in the UK (Tchilian *et al.*, 2001), and there is a debated association of the variant allele with multiple sclerosis (Jacobsen *et al.*, 2000; Vorechovsky *et al.*, 2001; Ballerini *et al.*, 2002; Miterski *et al.*, 2002). Jacobsen *et al.*, (2002) described another transversion mutation in exon 4 (C59A), which presents a similar T-cell phenotype to that observed in individuals with the C77G polymorphism. This appears to be extremely rare, being found in one of 311 MS patients in the study and not elsewhere.

We recently identified the first common CD45 variant with an allele frequency of 23.7% in the Japanese population and China (E.Tchilian, Personal communication). As discussed in chapters 3 and 5, the 138G variant allele promotes splicing on T cells to the low molecular weight CD45R0 isoform. In cohorts of Japanese patients, the 138G variant allele has a dominant protective effect in Graves' disease, and G138G homozygous individuals were found to be protected against Hashimoto's thyroiditis, both thyroid autoimmune diseases. We have also observed a protective effect against Hepatitis B infection (Boxall *et al.*, 2004).

Other recently described polymorphisms in the PTPRC gene are C77T (Pro59Pro) in exon 4, G69C (Asp121His) in exon 5, and T127A (Ile187Asn) in exon 6 (Gomez-Lira *et al.*, 2003). These new polymorphisms were found in Northern Italy at low frequencies

(0-1%) and their effects on CD45 alternative splicing are currently unknown (Gomez-Lira *et al.,* 2003). The main focus of this chapter will be the characterisation of two novel exon 4 polymorphisms: A54G (Stanton *et al.,* 2004) and A32G (D.Pretorius, personal communication).

The A54G variant results in a semi-conservative amino acid substitution (Thr 19 Ala). This A54G variant has been identified in the Ugandan population, but is absent from Japanese, Korean, Malawian and UK populations. Given the previously described increased frequency of the C77G variant allele in HIV-infected individuals, the distribution of the A54G allele was investigated in a cohort of HIV seropositive and seronegative individuals in Entebbe, Uganda. There was an increased frequency of the A54G variant allele in HIV seropositive Ugandan individuals (6 out of 160 HIV seropositive individuals, compared to 1 out of 108 seronegative individuals) which is suggestive of it having an effect. However, because of the low sample numbers the difference was not statistically significant ($p=0.25$ by Fishers exact test). No detailed analysis of A32G distribution was performed. Since previously described exon 4 polymorphisms (C77G and C59A) have been shown to have an effect on CD45 alternative splicing, the aim of this chapter is to determine whether the A54G and A32G variant alleles also have an effect.

## 6.2 Objectives

Recently two novel polymorphisms have been described in the PTPRC gene; A54G in exon 4 (Stanton *et al.,* 2004), and A32G in exon 4 (D.Pretorius, personal communication). The objective of this chapter is to characterise the CD45 expression on T cells from HIV seropositive individuals with these novel CD45 polymorphisms, to determine whether they alter CD45 splicing.

## 6.3 Results

### 6.3.1 Phenotypic analysis of T cells from exon 4 A54G variant individuals

As with the C77G polymorphism, the A54G polymorphism is within the region of exon 4 which has shown to be involved in the regulation of alternative splicing (Lynch and Weiss, 2001; Rothrock *et al.,* 2003). To determine whether the A54G variant allele has an effect on alternative splicing of CD45, phenotypic analysis was carried out on PBMC from HIV seropositive Ugandan individuals. PBMC were obtained from three HIV seropositive individuals (one A54G variant and two A54A controls) as determined by sequencing (Figure 6.1). To determine the pattern of CD45 expression, the cryopreserved PBMC were stained with CD3, CD4 or CD8 against CD45R0 and CD45RA or CD45RB and CD45RA. Figure 6.2 shows the CD45 expression profiles of CD3+ T cells. Unlike individuals with the C77G variant allele, both the A54G and A54A HIV seropositive individuals show the presence of both single and double positive CD45R0+ cells (Figure 6.2a). The individual with the A54G variant allele does appear to have an increased proportion of CD45R0+ CD45RA+ double positive cells, with around 20% of double positive cells compared to around 5% in the two control individuals. Whilst this could be an indication of altered CD45 expression, it could also be a result of the viral infection as both the disease status and viral load will have an effect on the proportions of and phenotype of T cells. Similar patterns of expression were observed in both CD4 and CD8 T cells (data not shown). No statistically significant differences were observed in the CD45RA CD45RB expression profiles (Figure 6.2b). Further analysis on PBMC from healthy HIV seronegative individuals with the A54G variant allele will be necessary to determine whether this polymorphism has a real effect on the alternative splicing of CD45.

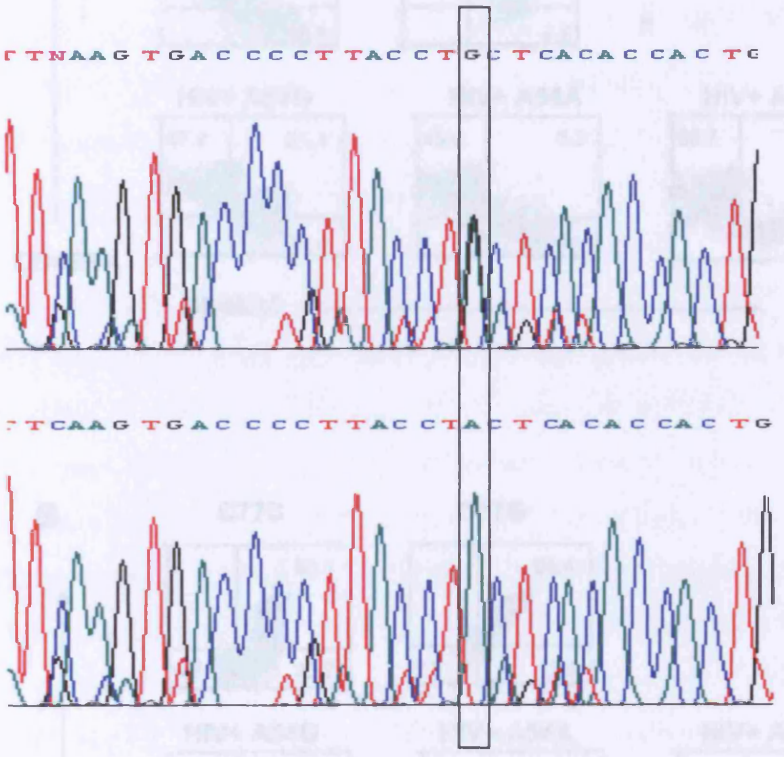



**Figure 6.1 The presence of the A54G polymorphism was confirmed by sequencing.** The box indicates the A to G transversion at position 54 of exon 4.

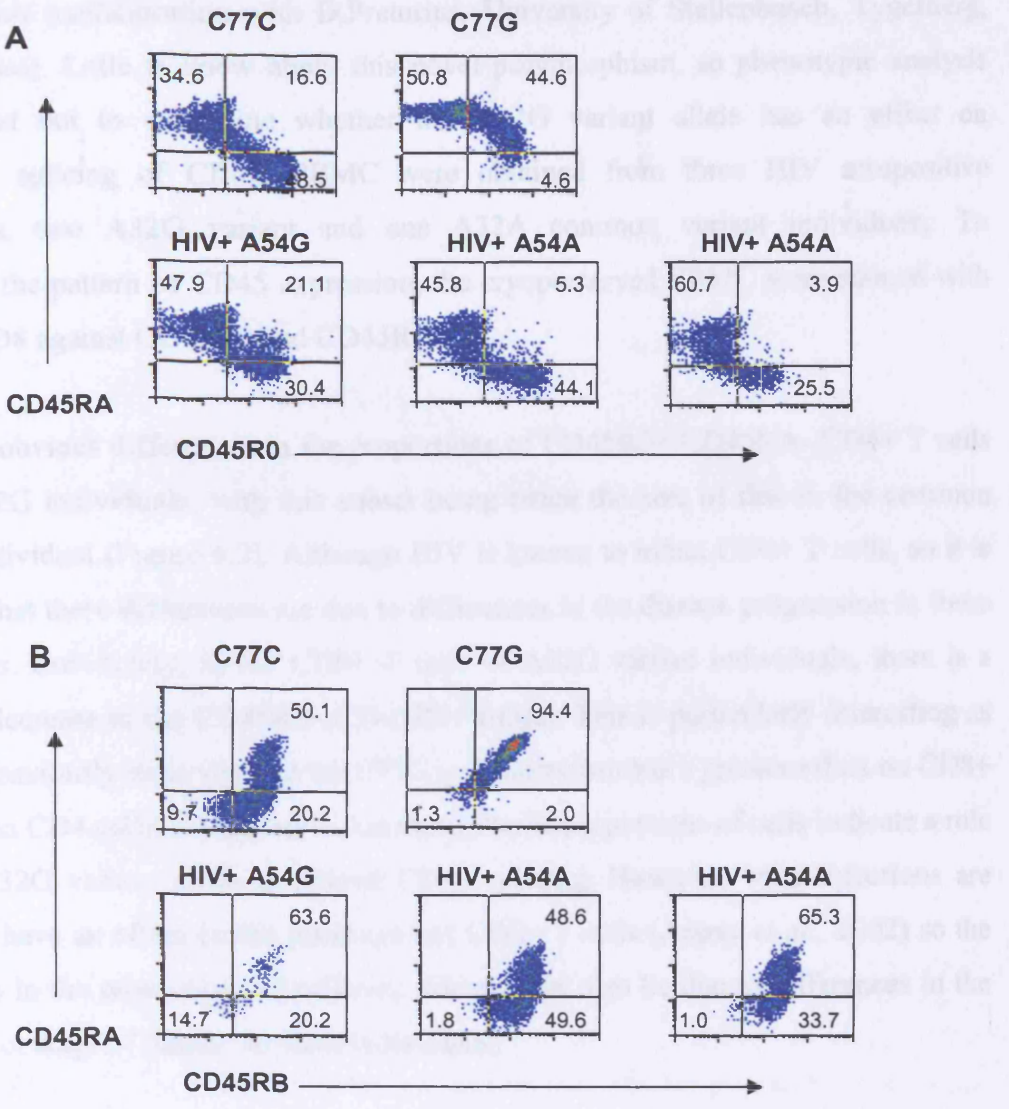



**Figure 6.2. Expression of CD45 isoforms in peripheral T cells in HIV seronegative European caucasoids with and without the C77G variant allele and Ugandan HIV seropositive individuals with and without the A54G variant allele.** PBMC samples were gated on CD3+ cells and expression of (A) CD45RA and CD45R0 and (B) CD45RA and CD45RB was analysed.

### 6.3.2 Phenotypic analysis of T cells from exon 4 A32G variant individuals

The exon 4 A32G polymorphism has been identified in HIV seropositive individuals in South Africa (collaboration with D.Pretorius, University of Stellenbosch, Tygerberg, South Africa). Little is know about this novel polymorphism, so phenotypic analysis was carried out to determine whether the A32G variant allele has an effect on alternative splicing of CD45. PBMC were obtained from three HIV seropositive individuals, two A32G variant and one A32A common variant individuals. To determine the pattern of CD45 expression, the cryopreserved PBMC were stained with CD4 or CD8 against CD45R0 and CD45RA.

There are obvious differences in the proportions of CD45R0+ CD45RA- CD4+ T cells in the A32G individuals, with this subset being twice the size of that in the common variant individual (Figure 6.3). Although HIV is known to infect CD4+ T cells, so it is probable that these differences are due to differences in the disease progression in these individuals. Conversely, in the CD8+ T cells of A32G variant individuals, there is a two-fold decrease in the CD45RA- CD45R0+ subset. This is particularly interesting as we have previously observed that the C77G polymorphism has a greater effect on CD8+ T cells than CD4 cells. It is possible that these altered proportions of cells indicate a role for the A32G variant allele in altered CD45 splicing. However, viral infections are known to have an effect on the phenotype of CD8+ T cells (Appay *et al.*, 2002) so the alterations in the proportions of different subsets may also be due to differences in the viral load or stage of disease in these individuals.

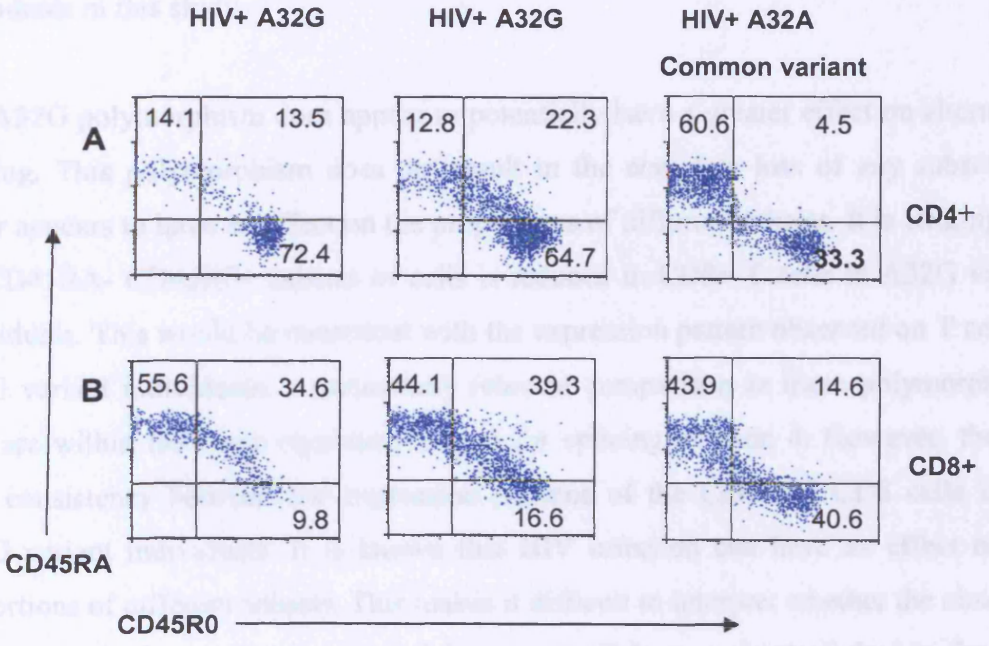



**Figure 6.3 Expression of CD45 isoforms in peripheral T cells in Ugandan HIV seropositive individuals with the A32G variant allele and A32A control.** PBMC samples were gated on (A) CD4+ helper cells or (B) CD8+ cytotoxic T cells and expression of CD45RA and CD45R0 was analysed.

## 6.4 Discussion

Given this preliminary data it appears unlikely that the A54G variant allele has a significant effect on splicing, and there is no abolition of a particular subset as is seen with the C77G polymorphism. It is possible that there are subtle alterations in the proportions of the different subsets, as is the case with the A138G polymorphism, but this would be difficult to distinguish due to the HIV seropositive status of the individuals in this study.

The A32G polymorphism does appear to potentially have a greater effect on alternative splicing. This polymorphism does not result in the complete loss of any subset, but rather appears to have an effect on the proportions of different subsets. It is striking that the CD45RA- CD45R0+ subsets of cells is reduced in CD8+ T cells in A32G variant individuals. This would be consistent with the expression pattern observed on T cells of C77G variant individuals, a particularly relevant comparison as these polymorphisms both are within the same regulatory region for splicing of exon 4. However, there is little consistency between the expression patterns of the CD4 and CD8 cells in the A54G variant individuals. It is known that HIV infection can have an effect on the proportions of different subsets. This makes it difficult to interpret whether the observed differences are due to the presence of the variant allele or a simply linked to the viral status of the individuals.

To determine the effect of both the A54G and A32G variant alleles on splicing, further analysis needs to be carried out on PBMC from healthy HIV-seronegative individuals with these polymorphisms. However, to determine the effect and exact mechanism of these polymorphisms, molecular studies utilising RT-PCR analysis or minigene constructs containing the mutation may be required.

It is interesting that both of these rare polymorphisms have been detected in HIV seropositive individuals. Whilst not statistically significant it is notable that the A54G variant allele was found at a higher frequency in the HIV seropositive sample group. It would be interesting to determine whether the frequency of the A32G allele is also altered in HIV seropositive individuals, or other viral infections. Further disease association studies will undoubtedly give insights into the subtle effects of these rare

polymorphisms and may give us a better understanding of the function of the CD45 isoforms.

# CHAPTER 7

## CD45 transgenic mouse models

### 7.1 Introduction

In humans, naïve T cells express high molecular weight exon A containing isoforms, but following activation switch to the expression of low molecular weight isoforms (CD45R0, and CD45RB low). A majority of memory cells maintain expression of CD45R0. CD45 isoform expression in mice is different, with few thymocytes or peripheral T cells expressing high molecular weight (CD45RABC) isoforms, whilst most express isoforms containing exon B. It is the level of CD45RB isoform expression, which separates functionally distinct T cells in mice and rats, with primed cells being CD45RB low, whilst CD45RB high cells are a mixture of both naïve and primed cells. B cells in both humans and rodents predominantly express high molecular weight (CD45RA containing) isoforms.

The simultaneous expression of multiple CD45 isoforms on leukocytes makes analysis of isoform function difficult. To overcome this issue several studies were carried out in which transformed cell lines were transfected with specific isoforms. These studies suggest that CD45 phosphatase activity is independent of the extracellular splice variant, with the cyoplamsic tail being sufficient to restore TCR signalling (Trowbridge, 1991; Hovis *et al.*, 1993; Volarevic *et al.*, 1993; Desai *et al.*, 1994). However, other studies have given contradictory results with either CD45RABC or CD45R0 being found to be more efficient at restoring TCR signalling (Novak *et al.*, 1994; Onodera *et al.*, 1996; Dornan *et al.*, 2002). This inconsistency is likely due to differences in the CD45 negative cell lines, stimuli and assays used. The interpretation of this data is further complicated in many studies by an inability to obtain physiological levels of isoform expression. This means that effects of the altered expression level may obscure any effects of isoform expression. Such limitations with cell line studies have lead to the production of CD45 knockout and transgenic mice.

Kishihara *et al.*, (1993) generated CD45 deficient mice by gene targeting of exon 6. B cell development appeared normal in these mice, although they failed to proliferate in response to IgM cross-linking. T cell development was blocked at the double positive stage, with the few mature T cells detected in the periphery unable to mount a CTL response to viral challenge. Whilst these mice suggested a role for CD45 in the development and function of T and B cells, endogenous CD45 expression was not completely abolished, making the effects difficult to interpret. To overcome this issue, Byth *et al.*, (1996) targeted exon 9 to create complete CD45 gene knockout mice. T cell development in these CD45-null mice was inhibited at both the double positive and single positive stages, leading to reduced numbers of mature T cells in the periphery and suggesting a role for CD45 in the regulation of signals through both the pre-TCR and TCR. These mice were also found to have increased numbers of IgM high splenic B cells and impaired B cell functional activity.

To determine the function of single CD45 isoforms, CD45 transgenic mice were created. These mice expressed single CD45RABC and CD45R0 isoforms under the control of the proximinal $p56^{lck}$ promoter (Kozieradzki *et al.*, 1997). This gave good expression in the thymus, and showed that both isoforms could restore thymic development. However, these CD45 trangenic mice were backcrossed onto exon 6 targeted knockout mice, which express some endogenous CD45 isoforms containing exons 4 and 5, and there was a ten fold difference in expression level between the CD45R0 and CD45RABC isoforms, making the isoform function difficult to interpret. To overcome this issue, Ogilvy *et al.*, (2003) created transgenic mice expressing single CD45RB and CD45R0 isoforms, backcrossed onto exon-9 targeted CD45-null mice. In this study the transgenes were under the control of vav1 gene transcriptional regulatory elements, so were expressed specifically throughout the hemopoietic compartment. Both isoforms were found to restore thymocyte development and peripheral T cells function in the CD45-null mice, with the expression level appearing to be the critical factor. However, even at high expression levels neither isoform was capable of fully restoring peripheral B cell maturation, suggesting that the role of the CD45 isoforms may differ between B and T cells.

To study the role of CD45 isoforms on peripheral T and B cells we introduced murine CD45RABC and CD45R0 cDNA trangenes (Tg) under the control of the human CD2 promotor into exon-9 targeted CD45 knockout (CD45-/-) mice (Figure 7.1). This generated CD45RABC Tg+ CD45-/- (CD45RABC/KO) and CD45R0 Tg+ CD45-/- (CD45R0/KO) mice (Tchilian *et al.*, 2004). These mice show that high-level transgene expression of either isoform can restore thymic differentiation, peripheral T cell function and immune responses to influenza virus and LCMV. However, as with the previous study, it is the expression level, which is critical for these processes.

Measuring T and B cell turnover shows increased CD8 cell proliferation and a block in B cell development in both the CD45RABC/KO and CD45R0/KO mice. Despite high CD45 expression levels entry to or survival of T cells in the spleen also appears to be compromised, resulting in reduced numbers and suggesting that either combinations of isoforms or even higher expression of a single isoform may be required. Peripheral T cells from these CD45 Tg mice have been found to have an activated phenotype, with higher CD44 and CD11a and lower CD62L expression. Whilst numbers and proliferation of peripheral B cells appears normal, they are found to have an immature phenotype in both CD45RABC/KO and CD45R0/KO mice. This would suggest that normal T and B cell differentiation and homeostasis requires not only a certain level of CD45 expression, but may also require the expression of particular combinations of isoforms.

From our human data we have observed that individuals with either the exon 4 C77G or exon 6 138G polymorphism have increased proportions of T cells with a memory / activated phenotype. Because of the striking observation that both humans with CD45 polymorphisms and CD45 Tg mice have increased proportions of activated/memory T cells in the periphery, we decided to use the transgenic mice as a model for the C77G human polymorphism. C77G heterozygous individuals have one CD45 allele capable of normal splicing, but the second allele is unable to splice to the low molecular weight isoforms. This results in constitutive CD45RA expression on T cells from variant individuals, even after stimulation. To model this situation we crossed CD45RABC/KO mice (CD45RABC Tg+ CD45-/-) with wild type (CD45+/+) mice, creating CD45RABC/WT mice, which have one fixed and one normally spliced allele. These

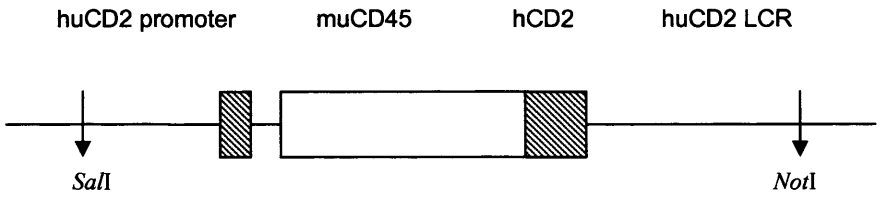



**Figure 7.1 Transgenic constructs.** The VAhCD2 cassette was used to generate CD45RABC and CD45R0 mice. The CD45cDNAs were inserted into the second exon of the hCD2 minigene, leaving the promoter and first hCD2 exon (depicted by shading) intact. As a poly A signal a 500 bp fragment from the 3' untranslated region of the hCD2 gene was used (shaded box). The expression cassette was separated from the plasmid with *Sal* I and *Not* I for microinjection.

mice were then used, in parallel with the human studies to investigate the effect of variant high molecular weight isoform expression on T cell phenotype and function.

## 7.2 Objectives

To use CD45 transgenic mice expressing one fixed CD45RABC and one wild type allele, as a model for the human C77G polymorphism, and analyse the effect of variant isoform expression on T cell phenotype and function.

## 7.3 Results

The data presented in this chapter is part of an ongoing collaborative project with Dr E. Tchilian and Mrs R. Dawes.

One of the major limitations in comparing the transgenic mouse data with that obtained from human studies is the difference in total CD45 expression level. In C77G variant individuals, total CD45 expression on T cells, monocytes and B cells was found to be equivalent to or slightly higher than that of the C77C controls. Despite having high and low expressing lines of CD45RABC/KO mice, even in high expressing lines the total CD45 expression is 3 to 5 times lower than that of the wild type mice.

As crossing CD45RABC/KO mice with wild type mice created the CD45RABC/WT Tg mice, it follows that the total CD45 expression in these mice is not equivalent to wild type mice. Therefore, to determine that any effects are due to the difference in isoform expression and not expression level, hemizygous (CD45+/-) mice were created as a control, by crossing wild type and CD45 knockout mice. In the following experiments these mice were also compared to wild type (CD45+/+), CD45 exon-9 targeted knockout (CD45-/-) and CD45RABC/KO (CD45RABC Tg+ CD45-/-) mice. To establish total CD45 expression level thymus, lymph node and spleen cells were stained with a murine panCD45 antibody, an antibody against an epitope present on all CD45 isoforms (Figure 7.2). Despite having theoretically higher total CD45 expression levels than the hemizygous controls, the CD45RABC/WT mice actually appear to have lower levels, particularly on lymph node T and B cells, likely due to an effect of the transgene and antibody reactivity.

The CD45 knockout mice have a partial block in thymic development, both in the transition from double negative to CD4+ CD8+ double positive cells and in the maturation of double positive to single positive CD4+ and CD8+ thymocytes. Introduction of the CD45RABC Tg (CD45RABC/KO) restores CD4+ thymocyte development to near normal levels. In hemizygous (CD45+/-) and CD45RABC/WT mice, thymocyte development is completely restored, with normal proportions and numbers of both CD4 and CD8 cells in the periphery (Figure 7.3). This shows that not

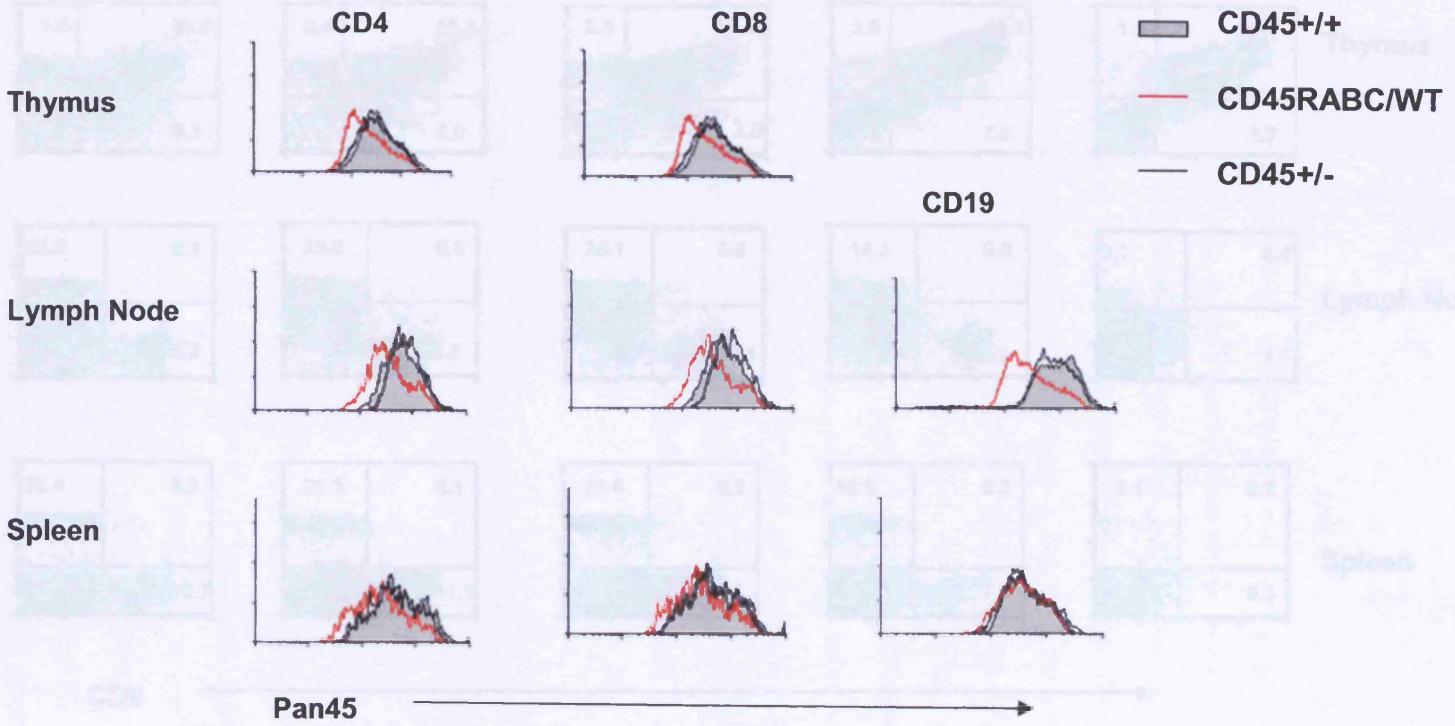



**Figure 7.2 Total CD45 expression on T and B cells in the thymus, lymph node and spleen from wild type (CD45+/+), CD45RABC/WT and hemizygous (CD45+/-) mice.** Flow cytometric analysis showing the surface expression of CD45 detected with a pan specific anti-CD45 monoclonal antibody on CD4, CD8 and CD19 gated cells from wild type (filled histogram), CD45RABC/WT (red line) and hemizygous (black line) mice. Data representative of three similar analyses.

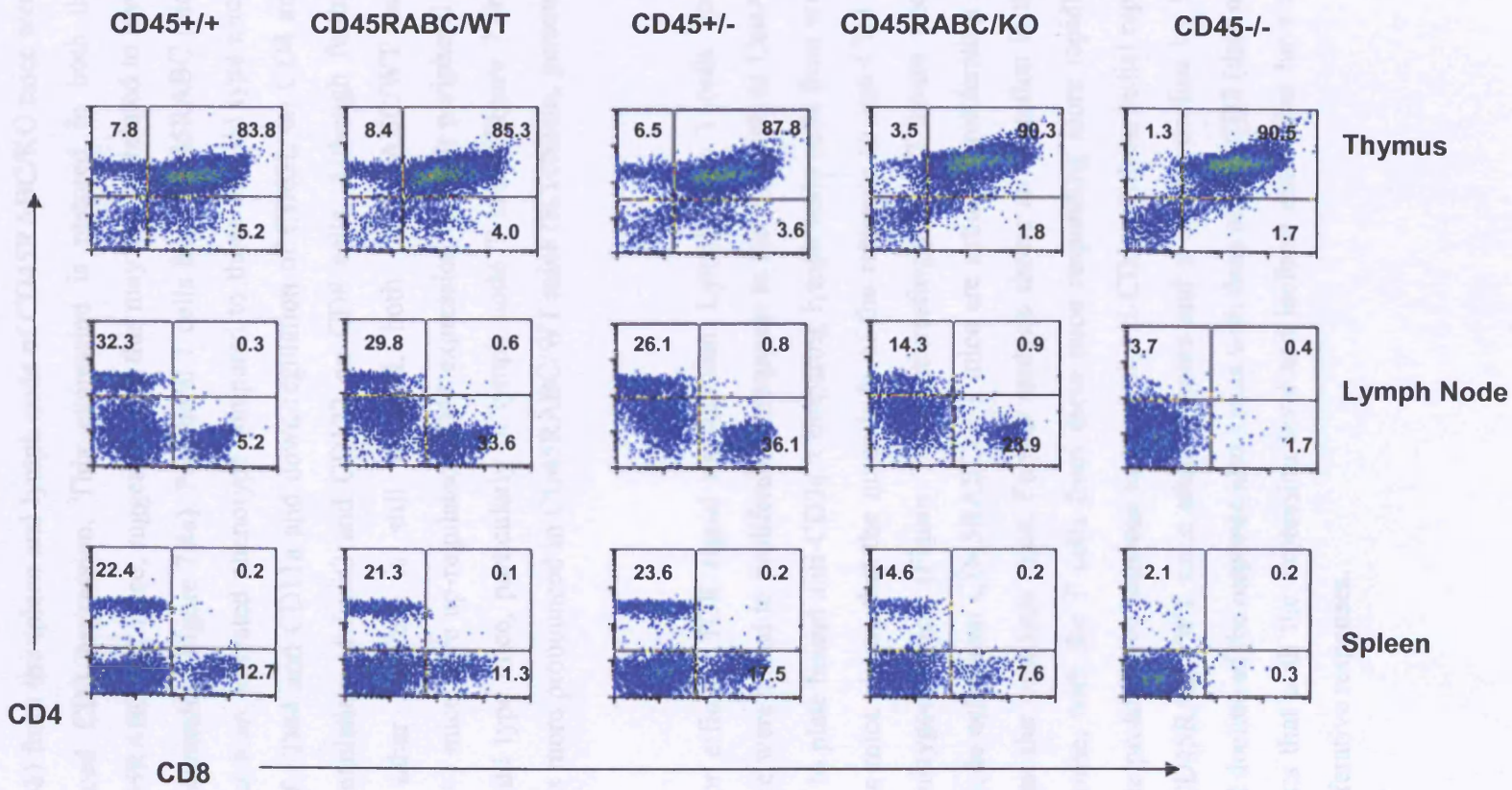



**Figure 7.3 Flow cytometric analysis of thymus, lymph node and spleen cells from wild type (CD45+/+), CD45RABC/WT, hemizygous (CD45+/-), CD45RABC/KO and CD45-/- mice.** Lymphocytes from the thymus, lymph nodes and spleen were double stained with anti-CD4 and anti-CD8 monoclonal antibodies. Data shown is representative of four similar analyses.

only is expression level important, but that increased expression of the high molecular weight isoforms does not have an effect on the proportions of peripheral T cells.

T cells (CD4 and CD8) from the spleen and lymph node of CD45RABC/KO mice were found to have reduced CD3 expression. This expression is restored in both the hemizygous and CD45RABC/WT mice, suggesting that this may also be related to level rather than isoform expression (Figure 7.4a). Peripheral T cells from CD45RABC mice were also found to have an activated phenotype compared to those of wild type mice, with up-regulation of CD44 and CD11a and down-regulation of CD62L on CD4 and CD8 cells, and up-regulation of Ly6C and CD122 on CD8 cells. Although further characterisation of other markers is still required, both CD45RABC/WT and hemizygous (CD45+/-) mice have up-regulated CD44 expression on their peripheral T cells compared to wild type mice, particularly in lymph node T cells (Figure 7.4b). However, the effect is more pronounced in CD45RABC/WT mice (E.Tchilain, personal communication).

CD45 is essential for efficient TCR signal transduction. Lymph node T cells from CD45RABC/KO mice were found to proliferate in response to lectins (such as ConA), but failed to respond to plate bound anti-CD3ε. Comparing lymph node cells from wild type and hemizygous mice shows that the magnitude of the response to anti-CD3 is reduced in the hemizygous mice (Figure 7.5a). Interestingly at 72 hours post-stimulation, lymph node cells from CD45RABC/WT mice are actually proliferating as well, if not better than the wild type mice. Further analysis shows an alteration in the kinetics of this response, with the T cells from these mice responding more rapidly. Figure 7.5b shows the proliferative response to 1 μg/ml anti-CD3ε, with an initial rapid response from the CD45RABC/WT mice after 24 hours and a swifter decline by 96 hours. The speed and decline of the response also varies with dose of anti-CD3 (data not shown). This suggests that both the expression level and isoform expression have an effect on T cell proliferative responses.

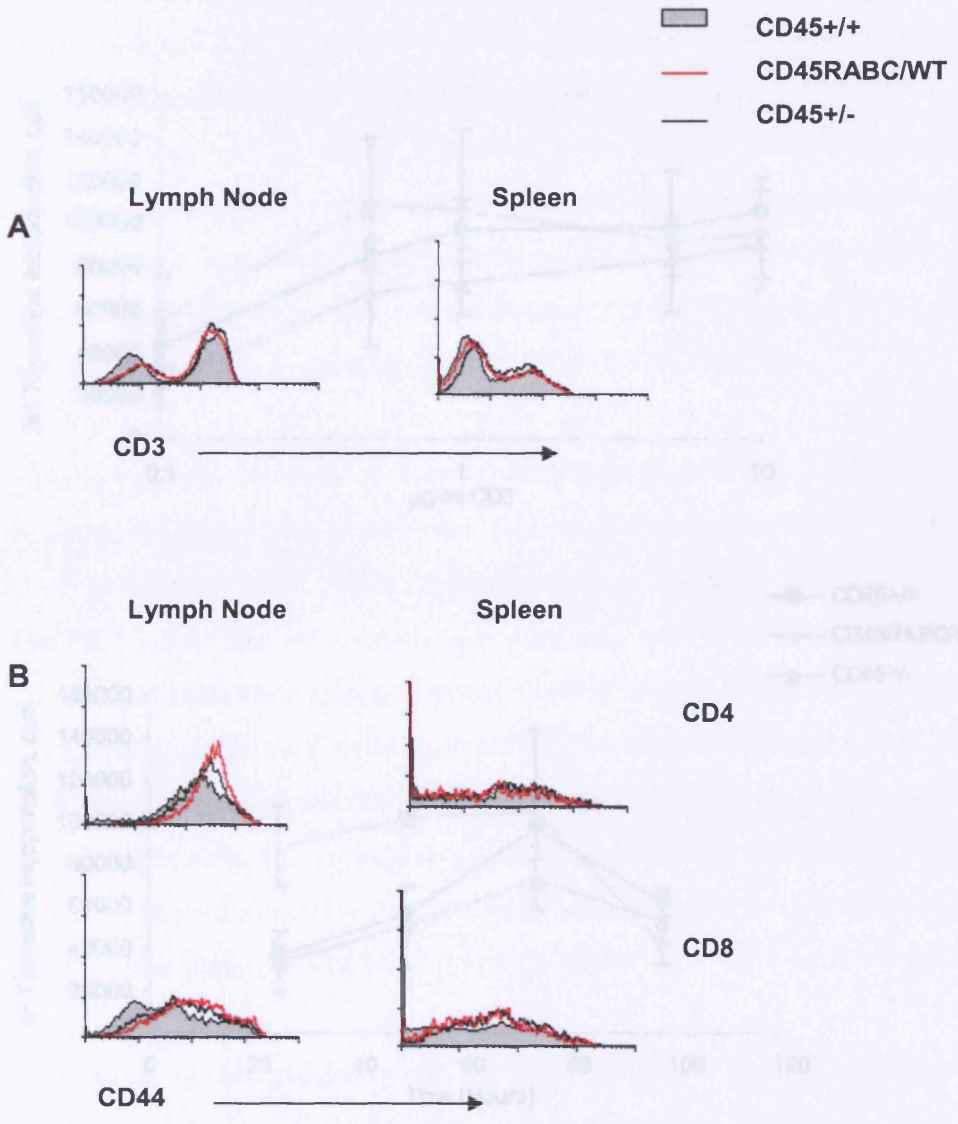



**Figure 7.4 Characterisation of T cells from the lymph node and spleen of wild type (CD45+/+), CD45RABC/WT and hemizygous (CD45+/-) mice.** Lymphocytes from the lymph nodes and spleen from wild type (filled histogram), CD45RABC/WT (red line) and hemizygous (black line) mice were stained with (A) anti-CD3 and (B) anti-CD4 or anti-CD8 and anti-CD44 monoclonal antibodies. Data shown is representative of four similar analyses.

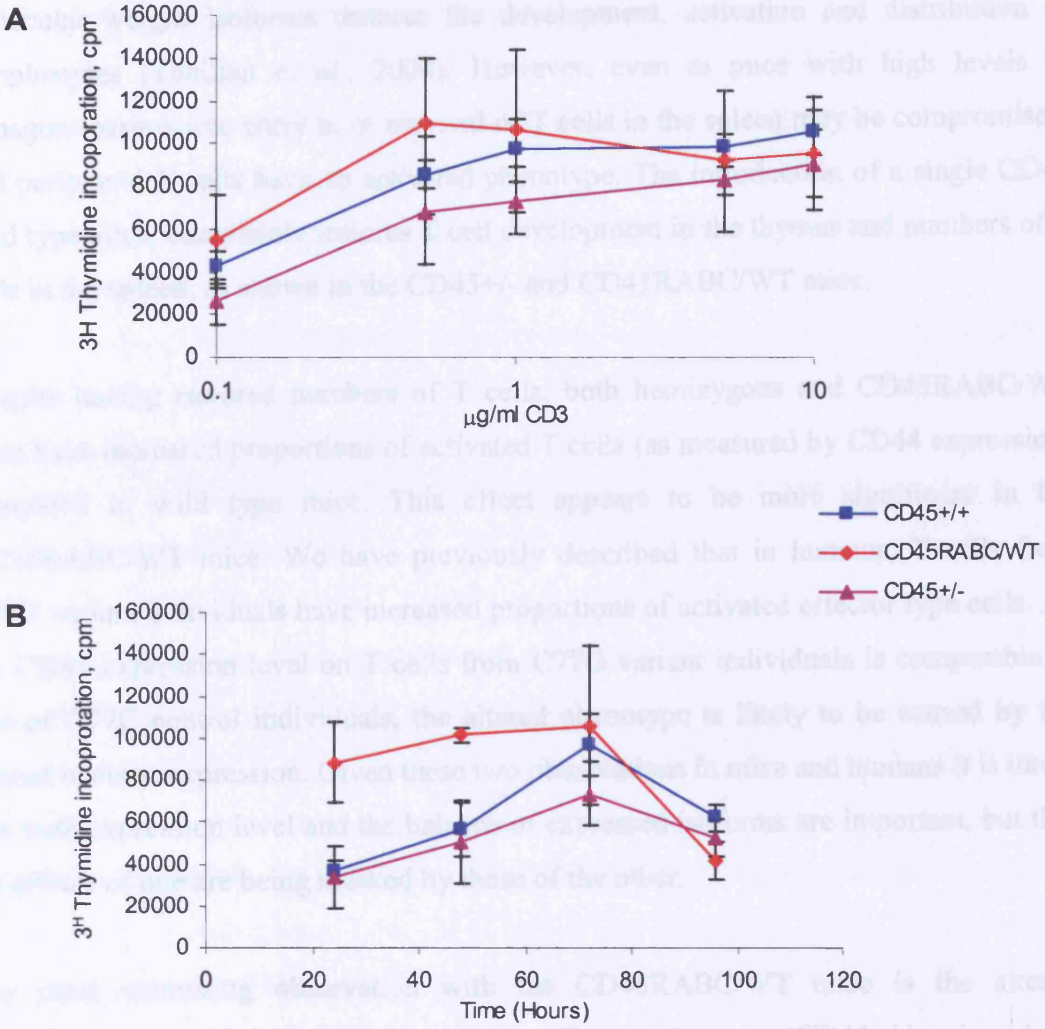



**Figure 7.5 Proliferative responses of lymph node cells from 3 wild type (CD45+/+), 3 CD45RABC/WT and 3 hemizygous (CD45+/-) mice.** Lymph node cells from CD45+/+, CD45RABC/WT and CD45+/- mice were stimulated with plate bound anti-CD3 antibody. Responsees were assayed by thymidine incorporation. Results are given in counts per minute (cpm) with each sample being assayed in triplicate. Each line represents the mean of three mice, corrected for background, with bars giving the standard deviation. (A) Response to varying doses of anti-CD3 at 72 hours. (B) Response to 1µg/ml anti-CD3 at varying time points. Data provided by Mrs R. Dawes.

## 7.4 Discussion

Studies with transgenic mice expressing single CD45 isoforms clearly show that the total level of expression is important. High level expression of either high or low molecular weight isoforms restores the development, activation and distribution of lymphocytes (Tchilian *et al.*, 2004). However, even in mice with high levels of transgene expression, entry to or survival of T cells in the spleen may be compromised, and peripheral T cells have an activated phenotype. The introduction of a single CD45 wild type allele completely restores T cell development in the thymus and numbers of T cells in the spleen, as shown in the CD45+/- and CD45RABC/WT mice.

Despite having restored numbers of T cells, both hemizygous and CD45RABC/WT mice have increased proportions of activated T cells (as measured by CD44 expression) compared to wild type mice. This effect appears to be more significant in the CD45RABC/WT mice. We have previously described that in humans, T cells from C77G variant individuals have increased proportions of activated effector type cells. As the CD45 expression level on T cells from C77G variant individuals is comparable to that of C77C control individuals, the altered phenotype is likely to be caused by the altered isoform expression. Given these two observations in mice and humans it is likely that both expression level and the balance of expressed isoforms are important, but that the effects of one are being masked by those of the other.

The most interesting observation with the CD45RABC/WT mice is the altered proliferative response to CD3 stimulation. The hemizygous (CD45+/-) mice show reduced proliferative responses to CD3 stimulation at 72 and 96 hours compared to wild type mice. The CD45RABC/WT mice respond more rapidly, followed by a rapid decline or exhaustion. This response of the hemizygous mice shows that altered expression level does have an effect on the proliferative response. The CD45 expression level of the CD45RABC/WT and hemizygous mice is not identical, but the slight difference would not be expected to cause the observed alteration in kinetics of the response of the CD45RABC/WT mice, particularly as the proportions of T cells and CD3 expression levels are found to be the same in these mice. This indicates that the expression of the high molecular weight isoform is having an effect. Further analysis of the CD45RABC/WT mice would be required to determine the cause of this altered

proliferative response to CD3 stimulation, particularly whether these mice have increased numbers of activated cells, or if these T cells have a lower activation threshold, so accounting for the rapid initial response. It would be interesting to investigate the effect of the altered isoform expression on apoptosis, and to determine if this accounts for the rapid decline of the proliferative response.

Preliminary experiments have so far shown no significant difference in the cytokine production of whole lymph node cells from wild type (CD45+/+), hemizygous (CD45+/-) and CD45RABC/WT mice in response to PMA and Ionomycin stimulation (data not shown). Given the altered proliferative responses to CD3, T cell cytokine production after CD3 stimulation is an interesting area for further study. Given that B cell maturation has previously been suggested to be dependant upon both expression level and the combinations of isoforms expressed (Tchilian et al., 2004), this would present an interesting area to investigate further, both in the CD45RABC/WT mice and humans with CD45 variant alleles.

Presently the mechanisms underlying the altered phenotype and function of lymphocytes of both humans with variant CD45 alleles and transgenic mice with altered expression of CD45 isoforms at the cell surface remain obscure. The CD45RABC/WT mice clearly show that both the expression level and isoform expression have an effect, but with the present model it is difficult to separate the effects of these two factors. The creation of mice with normal levels of CD45 surface expression, but an altered balance of different isoforms (such as single CD45RABC or CD45R0 expression) will provide a model to investigate the biochemical events leading to the observed altered phenotype and function of lymphocytes. Such a model would also provide an invaluable method of determining the immunological mechanisms underlying the disease associations of CD45 variant alleles in humans.

# CHAPTER 8

## Discussion

CD45 is a haemopoietic cell specific tyrosine phosphatase essential for efficient antigen receptor signal transduction in lymphocytes. CD45 is expressed in multiple isoforms in a cell type, differentiation and activation-specific manner. However the exact function of the different isoforms and the mechanism linking their expression to different leucocyte functions remains unclear. In spite of this lack of understanding of CD45 isoform function, it is clear that altered CD45 expression has major effects in humans. Lack of CD45 expression is a cause of severe combined immunodeficiency (SCID) and several point mutations of CD45 that cause altered splicing have been described and shown to be associated with infectious and autoimmune diseases.

The main objective of this project was to determine how the altered expression of alternatively spliced CD45 isoforms affects the function of lymphocytes. Utilising the existence of human CD45 polymorphisms (exon 4 C77G and exon 6 138G) we have studied the effect of altered CD45 isoform expression on T cell phenotype and function.

### The exon 4 C77G polymorphism

The C77G polymorphism is a point mutation in an exonic splice silencer which prevents splicing out of exon 4 (Thude *et al.*, 1995). T cells from variant individuals constitutively express high molecular weight CD45RA containing isoforms, even after stimulation. C77G heterozygous individuals are relatively rare (allele frequency of 0-2.3% in Europe, Asia and North America) and no homozygous individuals have been described. We have shown that whilst these individuals have normal proportions of different cell types, they have altered proportions of different T cell subsets, predominantly an increase in the proportion of CD8+ CD45RA+ effector cells. Such primed CD45RA+ cells are usually viral specific cells, often associated with CMV seroconversion, and are activated cells which have re-expressed CD45RA (Appay *et al.*,

2002; Kuijpers *et al.*, 2003). The increased size of this subset in C77G individuals may reflect a failure to efficiently deal with chronic viral infections. As this primed CD45RA+ population also increases with age (Hong *et al.*, 2004), the variant and control individuals used in this study were age matched where possible, but larger matched sample groups should ideally be used.

Given the postulated association of the C77G polymorphism with viral infections and autoimmune diseases such as MS, HIV, HCV, autoimmune hepatitis and systemic sclerosis, it has been suggested that these individuals may be more susceptible to or less efficient at clearing certain viral infections (Jacobsen *et al.*, 2000; Tchilian *et al.*, 2001; Ballerini *et al.*, 2002; Schwinzer *et al.*, 2003; Vogel *et al.*, 2003). Another striking example of this is the increased frequency of C77G variant individuals who were unable to clear the hepatitis C virus. This inability to clear chronic viral infection raises some interesting questions, particularly with regard to the altered T cell phenotype in variant individuals. It is unclear whether it is the presence of the variant allele which affects the T cell phenotype, or if the altered phenotype is a result of the infection.

This would make virus specific responses a key area of further study to establish whether the larger primed CD8+CD45RA subset is a revertant cell population and if it is CMV specific. Tetramers could be used to determine whether these are primed cells specific to dominant CMV epitopes, and heteroduplex analysis could be used to detect expanded clones within the subset (Maini *et al.*, 1998; Wills *et al.*, 2002). However, susceptibility to or inability to clear viral infection is unlikely to be purely due to an alteration in the proportions of T cell subsets, so other cell types, such as B cells, NK cells or APCs should also be analysed. Given the increased total CD45 expression observed on monocytes from C77G variant compared to control individuals, this may be a particularly relevant cell type for further analysis. Furthermore NK cells have recently been shown to have a role in anti-HCV immune responses (Khakoo *et al.*, 2004), making NK cell cytotoxicity another potential area of further investigation.

T cells from C77G variant individuals were found to have reduced proliferative response to CD3, whilst their responses to soluble recall antigens such as tetanus toxoid and PPD appear normal. As primed CD45RA+ cells have been shown to respond poorly to CD3 (Appay *et al.*, 2002), the reduction in the proliferative response may be a

reflection of the increased proportion of these cells in C77G variant individuals. To date all of the proliferative responses have been measured in PBMC cultures. Therefore the mitogenic responses of specific T cell subsets could be further dissected using CSFE labelling. As CD45 isoform expression alters with time following mitogenic stimulation, thymidine incorporation assays using purified cell subsets (CD45RA+ or CD45R0+ cells) may also be required to follow the proliferative responses at later time points.

As the increased proportion of CD45RA+ primed cells is predominantly observed in the CD8 population, the antigen specific responses of this subset also needs to be investigated. Possible areas of study could include allo, influenza and EBV-specific CTL responses, to determine whether the function of the CD8+ CD45RA+ cells have been altered in C77G variant individuals. In addition to assays for general immune competence it would also be interesting to determine whether CD45 variant individuals have altered responses to disease antigens, with both HBV and HCV being likely candidates for study.

**The exon 6 138G polymorphism**

The second polymorphism analysed in this study is the exon 6 138G polymorphism. This is the first common CD45 variant to be described in humans, with nearly 40% of the Japanese population being heterozygous and 5.1% homozygous for the G allele (Stanton *et al.*, 2003). This polymorphism results in an amino acid substitution (Threonine 47 to Alanine) and interferes with alternative splicing. By analysing CD45 mRNA expression in PBMC from CD45 variant individuals and using minigene constructs transfected in COS-7 cells, we have demonstrated that the 138G variant allele promotes splicing towards the low molecular weight isoforms. The 138G variant individuals therefore have increased proportion of CD45R0+ T cells. We have shown that T cells from these individuals contain increased proportions of the highly differentiated central memory subset (CD8+ CD45R0+ CCR7-).

Cohorts of patients with Graves' disease (an autoimmune thyroiditis) or HBV infection have reduced number of 138G variant individuals compared to controls, suggesting a

strong protective effect of the 138G allele (Boxall *et al.*, 2004). The underlying mechanisms of this effect are unknown. However, compared to A138A common variant individuals, both A138G heterozygous and G138G homozygous individuals have increased proportions of T cells capable of producing IFNγ *in vitro*. In Graves' disease there is a shift towards a Th2 cytokine response, which could be counteracted by the increased IFNγ production in 138G variant individuals (Uchimura *et al.*, 2002). Similarly the anti-viral effects of IFNγ have also been shown to be a protective mechanism in HBV infection (Guidotti and Chisari, 2001).

The high frequency of the 138G variant allele in Japanese, Chinese, Thai and Cambodian populations (unpublished data) suggest that it may also affect susceptibility and pathogenesis in other autoimmune or infectious disease and could have an important effect on disease incidence in a large proportion of the human population. The increased proportion of IFNγ producing T cells in 138G variant individuals and the apparent protective effect of the variant allele in Graves' disease, a type 2 autoimmune thyroiditis, suggest that the variant allele may have an influence in diseases where the Th1/Th2 balance is important. This might include conditions such as allergy and helminth infections.

The presence of increased proportions of IFNγ producing T cells in 138G variant individuals raises an interesting question, as to whether this is a specific effect on IFNγ or whether the expression of other cytokines is also affected. If it is a general effect, then is there an up-regulation of all Th1 and Th2 cytokines or it is there a bias towards production of Th1 type cytokines, such as IFNγ ? We were not able to detect differences in the amount of other cytokines (IL-2, IL-4, IL-5, IL-10, TNFα) produced by T cells from 138G variant and A138A control individuals, suggesting that this is a specific effect on IFNγ production. However, as there can be a difference between the proportions of cells capable of producing cytokines and the amount produced may be dependant upon the stimulus used, more detailed analyses are required. Polarisation of naïve cells to Th1 or Th2 could be investigated using cytokine blocking antibodies, to determine if cells from 138G variant individuals are more easily biased towards a Th1 response (Messi *et al.*, 2003). Quantification of expression levels of fate-determining T-

bet and GATA-3 transcription factors could also be used as an indication of T cell commitment to either the Th1 or Th2 lineage.

CD45 regulates antigen receptor signalling by dephosphorylation of Src kinases and can modulate cytokine receptor signalling via Janus Kinases (Jaks) (Irie-Sasaki *et al.*, 2001; Hermiston *et al.*, 2003). Given the altered proliferative response to CD3 stimulation observed in both CD45 variant individuals and transgenic mice, it would be logical to investigate any possible alterations in TCR signalling. Such studies may include $Ca^{2+}$ mobilisation assays and measuring the phosphorylation status and kinase activities of p56lck and p59fyn. Since 138G variant individuals show increased IFNγ production and CD45 knockout mice have been shown to have increased cytokine production, with corresponding hyperphosphorylations of Jak 2, Stat 3 and Stat 5 in different cell types (Irie-Sasaki *et al.*, 2001), future studies should also determine the kinetics and magnitude of phosphorylation events downstream of the cytokine receptors. The basal phosphorylation status of Jaks should be measured in various subsets of cells from 138G variant and A138A control individuals, whilst Stat3 and Stat5 phosphorylation should be assessed following activation with cytokines.

**Other CD45 polymorphisms**

Several other rare CD45 polymorphisms have been described at frequencies of 0-1%, but no information is currently available as to their effects on alternative splicing or associations with disease (Gomez-Lira *et al.*, 2003). The exon 4 C59A mutation has been shown to cause abnormal CD45 splicing, but has so far only been described in one MS patient and not elsewhere (Jacobsen *et al.*, 2002). Although not fully characterised at present, the novel exon 4 (A54G) polymorphism described in African Ugandan populations, is present with increased frequency in HIV seropositive individuals (Stanton *et al.*, 2004). Screening for novel polymorphisms in other regions of the CD45 molecule and ethnic distribution studies of the different polymorphisms may provide a basis for future and more detailed disease association studies.

**CD45 transgenic mice**

Transgenic mice have previously been constructed which express single high (CD45RABC) or low (CD45R0) molecular weight isoforms on a CD45 knockout background under the control of the human CD2 promoter (Tchilian *et al.*, 2004). Phenotypic analysis and *in vivo* challenge of these mice with influenza and LCMV shows that T cell differentiation and peripheral T cell function are related to the level of total CD45 expression rather than isoform. These mice were found to have an increased proportion of activated cells and altered proliferative and cytokine responses. It is interesting that both in individuals with the C77G and A138G polymorphisms and CD45 transgenic mice, CD8 cells appear to be more affected by abnormal CD45 expression than CD4 cells. This could be indicative of differential requirements of CD45 isoform expression during the development of and signalling events in CD4 and CD8 cells. Furthermore mice expressing high levels of transgene were found to have a block in B cell maturation (Ogilvy *et al.*, 2003; Hong *et al.*, 2004) suggesting a role for the expression of combinations of isoforms. As there are a number of parallels in the data from the transgenic mice and CD45 variant individuals, further characterisation of B cells in CD45 variant individuals may prove an interesting area of further study.

We created a Tg mouse model for the human C77G polymorphism, by crossing transgenic mice expressing single CD45RABC isoform on a CD45 knockout background with wild type mice. These CD45RABC/WT mice have one normally spliced allele and one high molecular weight allele which can not be spliced, so mimicking the pattern of CD45 isoform expression observed on T cells from C77G variant individuals. Data from the CD45RABC/WT mice suggests that both expression level and isoform expression are important in T cell phenotype and function. These mice have increased proportions of activated (CD44 high) T cells and altered proliferative response to CD3 stimulation. There is a distinct shift in the kinetics of the CD3 response, with cells from CD45RABC/WT mice responding faster and declining quicker than either the wild type or hemizygous (CD45+/-) mice.

The CD45RABC/WT mice show that both the expression level and isoform expression have an effect on T cell phenotype and function, but at present it is difficult to separate the effects of these two interrelated factors. Generation of Tg mice with normal levels of

CD45 surface expression, but an altered balance of different isoforms will provide a model to investigate the underlying mechanisms of altered phenotype and function of lymphocytes, and of disease associations. Such Tg mice would be an invaluable model for investigating the *in vivo* effects of variant CD45 isoform expression on viral infection and autoimmune disease.

In summary, in this study we have shown that altered CD45 isoform expression affects T cell phenotype and function. Two different human CD45 variants with contrasting phenotypes and disease associations were analysed. The exon 4 C77G polymorphism leads to constitutive CD45RA expression, with increased proportions of primed CD8+ CD45RA+ cells, and reduced proliferative responses to CD3 stimulation. This polymorphism is found at higher frequencies in some autoimmune and infectious diseases. In contrast the exon 6, 138G variant allele, promotes splicing towards low molecular weight CD45R0 isoforms, with variant individuals having increased proportions of central memory and IFNγ producing T cells. The 138G variant allele is associated with protection against Graves' disease and hepatitis B.

Although we still do not completely understand how the expression of the alternatively spliced isoforms affects lymphocyte functions, we have clearly shown that altered isoform expression both in CD45 variant humans and CD45 Tg mice can have profound effects on immune functions and diseases. Understanding how the expression of CD45 isoforms affects immune function is important, as it could lead to the development of therapeutic agents targeting CD45 and the signalling pathways that it modulates. It could also provide insights into the function of naive and memory cells, leading to new strategies for vaccination or immunomodulation.

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# *Appendix*

## *Publications arising from this work*

# A high-frequency polymorphism in exon 6 of the CD45 tyrosine phosphatase gene (*PTPRC*) resulting in altered isoform expression

Tara Stanton*, Sally Boxall†, Kouzo Hirai‡, Ritu Dawes†, Susan Tonks*, Tomoyo Yasui‡, Yasushi Kanaoka‡, Nadira Yuldasheva*, Osamu Ishiko‡, Walter Bodmer*, Peter C. L. Beverley†, and Elma Z. Tchilian†§

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BRIEF COMMUNICATION

Tara Stanton · Sally Boxall · Andrea Bennett ·
Pontiano Kaleebu · Christine Watera ·
Jimmy Whitworth · Neil French · Ritu Dawes ·
Adrian V. S. Hill · Walter Bodmer ·
Peter C. L. Beverley · Elma Z. Tchilian

# CD45 variant alleles: possibly increased frequency of a novel exon 4 CD45 polymorphism in HIV seropositive Ugandans

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PEDIATRIC RESEARCH

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# Abnormal Cell Surface Antigen Expression in Individuals with Variant CD45 Splicing and Histiocytosis

SALLY BOXALL, JAMES MCCORMICK, PETER BEVERLEY, STEPHAN STROBEL, PAOLA DE FILIPPI, RITU DAWES, CATHERINE KLERSY, RITA CLEMENTI, EMANUELLA DE JULI, ALINE FERSTER, DIANA WALLACE, MAURIZIO ARICÒ, CEZARE DANESINO, AND ELMA TCHILIAN

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# Disease associations and altered immune function in CD45 138G variant carriers

**Sally Boxall[1,†], Tara Stanton[2,†], Kouzo Hirai[3,†], Victoria Ward[2], Tomoyo Yasui[3], Hideki Tahara[3], Akihiro Tamori[3], Shuhei Nishiguchi[3], Susumu Shiomi[3], Osamu Ishiko[3], Masaaki Inaba[3], Yoshiki Nishizawa[3], Ritu Dawes[1], Walter Bodmer[2], Peter C.L. Beverley[1] and Elma Z. Tchilian[1,*]**